Extraction, Characterization and Storage Stability of Lipid from Hepatopancreas of Pacific White Shrimp (*Litopenaeus vannamei*)

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

Prince of Songkla University

2014

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

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ชื่อวิทยานิพนธ์ การสกัด การจำแนกลักษณะและความคงตัวระหว่างการเก็บรักษาน้ำมันจากตับอ่อนของกุ้งขาว

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บทคัดย่อ

จากการศึกษาการสกัด และการจำแนกลักษณะของลิปิดจากหัวและตับอ่อนของกุ้งขาวที่เก็บรักษาในน้ำแข็งเป็นเวลา 6 วัน พบว่า ผลผลิตการสกัดลิปิดจากตับอ่อนสูงกว่าผลผลิตจากส่วนหัวของกุ้งขาว เมื่อเวลาการเก็บรักษาลิปิดเพิ่มขึ้น สารไฮโดรเปอร์ออกไซด์มีปริมาณสูงขึ้น โดยบ่งชี้จากสเปคตราค่าการดูดกลืนแสงของ Fourier transform infrared (FTIR) ในช่วงคลื่น 3600 – 3200 cm^{-1} ที่สูงขึ้นและค่าเปอร์ออกไซด์ (PV) ที่สูงขึ้น ($p < 0.05$) นอกจากนี้ค่า thiobarbituric acid reactive substances (TBARS) ค่าพาราอะนิซิดีน (AnV) และกรดไขมันอิสระของลิปิดมีค่าเพิ่มขึ้น เมื่อระยะเวลาการเก็บรักษาในน้ำแข็งนานขึ้น ($p < 0.05$) ดังนั้นการเก็บรักษาวัตถุดิบเป็นระยะเวลานานขึ้นส่งผลให้เกิดการเสื่อมเสียของลิปิดที่เพิ่มขึ้น

เมื่อศึกษาผลของสภาวะการสกัดลิปิดและผลผลิตของแคโรทีนอยด์จากตับอ่อนของกุ้งขาว พบว่า ตัวทำละลายผสมระหว่างเฮกเซนและไอโซโพรพานอล (อัตราส่วน 50:50 โดยปริมาตร) ให้ลิปิดที่มีความเข้มข้นของแคโรทีนอยด์สูงสุด (336.40 มิลลิกรัมต่อกิโลกรัมตับอ่อน) และให้ผลผลิตการสกัดลิปิดร้อยละ 18.22 (โดยน้ำหนักของตับอ่อน) ($p < 0.05$) เมื่อใช้อัตราส่วนของตับอ่อนต่อตัวทำละลายเท่ากับ 1: 4.5 (โดยน้ำหนักต่อปริมาตร) และสกัดซ้ำ 3 ครั้ง ทำให้ได้ความเข้มข้นของแคโรทีนอยด์สูงสุด (363.94 มิลลิกรัมต่อกิโลกรัมตับอ่อน) และให้ผลผลิตการสกัดลิปิดร้อยละ 18.08 (โดยน้ำหนักของตับอ่อน) นอกจากนี้เมื่อเติมแอสตาแซนทีน (2 มิลลิกรัมต่อกรัมลิปิด) ในลิปิดที่สกัดได้ พบว่า ปฏิกิริยาออกซิเดชันของลิปิดลดลงเมื่อเปรียบเทียบกับตัวอย่างชุดควบคุมในระหว่างการเก็บรักษาที่ 30 องศาเซลเซียส เป็นเวลา 10 วัน ซึ่งบ่งชี้จากค่า AnV ที่ลดลง และจากสเปคตรา FTIR พบว่า ไตรกลีเซอไรด์ในลิปิดเกิดปฏิกิริยาออกซิเดชันในอัตราที่ต่ำเมื่อเติมแอสตาแซนทีนลงในลิปิด

เมื่อศึกษาความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันของลิปิดจากตับอ่อนของกุ้งขาว ที่เติมน้ำมันหอมระเหยชนิดต่างๆ ที่ความเข้มข้น 200 มิลลิกรัมต่อกิโลกรัม ระหว่างการเก็บรักษานาน 10 วัน พบว่า เมื่อเวลาการเก็บรักษาลิปิดเพิ่มขึ้น สารไฮโดรเปอร์ออกไซด์มีปริมาณเพิ่มขึ้น โดยบ่งชี้จากค่า PV ที่เพิ่มขึ้น ($p < 0.05$) เมื่อเปรียบเทียบน้ำมันหอมระเหยทั้งหมดที่ใช้

พบว่า น้ำมันหอมระเหยจากเปลือกมะนาวสามารถป้องกันการเกิดปฏิกิริยาออกซิเดชันได้สูงสุด ตามด้วยน้ำมันหอมระเหยจากไพล และใบกระเพรา ตามลำดับ อย่างไรก็ตามประสิทธิภาพในการป้องกันการเกิดปฏิกิริยาออกซิเดชันของน้ำมันหอมระเหยทั้ง 3 ชนิด ต่ำกว่าโทโคฟีรอลที่ระดับความเข้มข้นเดียวกัน สารประกอบที่ระเหยได้จากปฏิกิริยาออกซิเดชัน และกลิ่นหืนในลิปิดมีค่าลดลงเมื่อมีการเติมน้ำมันหอมระเหย ดังนั้นน้ำมันหอมระเหยสามารถใช้เป็นสารต้านปฏิกิริยาออกซิเดชันจากธรรมชาติเพื่อป้องกันการเกิดปฏิกิริยาออกซิเดชัน และกลิ่นหืนของลิปิดจากตับอ่อนระหว่างการเก็บรักษา

จากการศึกษาความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันในระบบอิมัลชันชนิดน้ำมันในน้ำ (พีเอช 3.6) ที่เติมสารต้านปฏิกิริยาออกซิเดชันชนิดต่างๆ ที่ระดับความเข้มข้นต่างกันระหว่างการเก็บรักษาที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 12 วัน พบว่า เมื่อเวลาการเก็บรักษาอิมัลชันเพิ่มขึ้น สารไฮโดรเปอร์ออกไซด์มีปริมาณเพิ่มขึ้น ซึ่งบ่งชี้จากค่า PV ที่เพิ่มขึ้น ($p < 0.05$) อิมัลชันที่เติมโทโคฟีรอลเข้มข้น 200 ppm มีค่า PV สูงกว่าอิมัลชันอื่นๆ ($p < 0.05$) นอกจากนี้ค่า TBARS ของทุกตัวอย่างเพิ่มขึ้นตลอดระยะเวลาการเก็บรักษา ($p < 0.05$) พร้อมกับมีสารประกอบที่ระเหยได้จากปฏิกิริยาออกซิเดชัน และกลิ่นหืนที่ลดลง อิมัลชันที่มีการเติมสารต้านปฏิกิริยาออกซิเดชันโดยเฉพาะอิมัลชันที่เติม EDTA เข้มข้น 50 ppm ร่วมกับกรดแทนนิกเข้มข้น 100 ppm หรือ EDTA เข้มข้น 50 ppm ร่วมกับกรดแทนนิกเข้มข้น 100 ppm เลซิตินเข้มข้น 500 ppm และโทโคฟีรอลเข้มข้น 200 ppm มีปฏิกิริยาออกซิเดชันที่ลดลง ดังนั้นการใช้ EDTA ร่วมกับกรดแทนนิกสามารถป้องกันการเกิดปฏิกิริยาออกซิเดชันในระบบอิมัลชันชนิดน้ำมันในน้ำได้อย่างมีประสิทธิภาพ เมื่อเปรียบเทียบกับการใช้สารต้านปฏิกิริยาออกซิเดชันอื่น

จากการเตรียมไมโครเอนแคปซูลน้ำมันกุ้งโดยใช้ส่วนผสมระหว่างเวย์โปรตีนและโซเดียมเคซีเนต (1: 1 โดยปริมาตร) เป็นสารห่อหุ้ม โดยศึกษาผลของอัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้ม (1: 1 และ 1: 2 โดยน้ำหนัก) และระดับแรงดันโฮโมจิไนซ์ (2,000 และ 4,000 ปอนด์ต่อตารางนิ้ว) ต่อลักษณะและความคงตัวของอิมัลชัน พบว่า ขนาดอนุภาคของอิมัลชันลดลงเมื่อระดับแรงดันโฮโมจิไนซ์สูงขึ้น ($p < 0.05$) แต่อัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้มไม่มีผลกระทบต่อขนาดอิมัลชัน ($p > 0.05$) เมื่อเวลาการเก็บรักษาอิมัลชันเพิ่มขึ้น ขนาดอนุภาค flocculation factor (F_f) และ coalescence index (C_i) ในอิมัลชันมีค่าเพิ่มขึ้น โดยเฉพาะอิมัลชันที่เตรียมโดยใช้แรงดันโฮโมจิไนซ์ที่ 2,000 ปอนด์ต่อตารางนิ้ว และใช้อัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้มเท่ากับ 1: 2 (โดยน้ำหนัก) ($p < 0.05$) ภายหลังการทำแห้งแบบพ่นฝอย พบว่า อนุภาคของไมโครแคปซูลน้ำมันกุ้ง (MSO) ที่เตรียมโดยใช้แรงดันโฮโมจิไนซ์ที่ 2,000 ปอนด์ต่อตารางนิ้ว และอัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้มเท่ากับ 1: 2 (โดยน้ำหนัก) มีขนาดอนุภาคที่ใหญ่กว่าอนุภาค MSO อื่นๆ ($p < 0.05$)

สำหรับ MSO ที่เตรียมโดยใช้แรงดันโฮโมจิไนซ์เท่ากับ 4,000 ปอนด์ต่อตารางนิ้ว และใช้อัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้มเท่ากับ 1: 4 (โดยน้ำหนัก) มีประสิทธิภาพในการห่อหุ้ม (encapsulation efficiency, EE) (ร้อยละ 51.3 - 52.8) สูงกว่า MSO อื่น ดังนั้น อัตราส่วนของน้ำมันกุ้งต่อสารห่อหุ้มและระดับแรงดันโฮโมจิไนซ์มีผลต่อ MSO

เมื่อศึกษาผลของสารห่อหุ้มที่แตกต่างกัน ได้แก่ เวย์โปรตีนเข้มข้น (WPC): โซเดียมเคซีเนต (SC) (1: 1 โดยน้ำหนัก) WPC: SC: กัมอาราบิก (1: 1: 2 โดยน้ำหนัก) WPC: SC: กลูโคสไซรัป (1: 1: 2 โดยน้ำหนัก) และ WPC: SC: มอลโตรเด็กตริน (1: 1: 2 โดยน้ำหนัก) ต่อกระบวนการเอนแคปซูเลชัน พบว่า สารห่อหุ้มผสมระหว่าง WPC SC และ กลูโคสไซรัปมีค่า EE สูงสุด (ร้อยละ 84.43 – 88.19) ขณะที่สารห่อหุ้มผสมระหว่าง WPC และSC ให้ค่า EE ต่ำสุด (ร้อยละ 50.37 – 53.05) เมื่อทำการศึกษาความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันของ MSO ที่ห่อหุ้มด้วย WPC SC และกลูโคสไซรัปร่วมกับการเติมสารต้านปฏิกิริยาออกซิเดชันต่างๆ ระหว่างการเก็บรักษาที่อุณหภูมิ 30 องศาเซลเซียส เป็นเวลา 6 สัปดาห์ พบว่า เมื่อระยะเวลาการเก็บรักษา MSO เพิ่มขึ้น ค่า PV เพิ่มขึ้น ($p < 0.05$) ซึ่ง MSO ที่เติมน้ำมันหอมระเหยจากเปลือกมะนาว ร่วมกับ EDTA และกรดแทนนิก มีค่า PV ต่ำสุดภายหลังการเก็บรักษานาน 6 สัปดาห์ ($p < 0.05$) ดังนั้น การใช้สารห่อหุ้มที่เหมาะสมร่วมกับการเติมสารต้านปฏิกิริยาออกซิเดชันทำให้ได้ MSO ที่มีความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันที่สูง

จากการศึกษาลักษณะและความคงตัวต่อการเกิดปฏิกิริยาออกซิเดชันของขนมปังที่เสริม MSO พบว่า การเติม MSO สามารถเพิ่มปริมาตรของขนมปัง แต่อย่างไรก็ตามค่า chewiness gumminess และresilience ของขนมปังมีค่าลดลง สำหรับสีของเปลือกและเนื้อขนมปังมีค่าสีแดงและค่าสีเหลืองสูงขึ้นเมื่อเติม MSO ลงในขนมปัง ($p < 0.05$) จากโครงสร้างจุลภาคของขนมปัง พบว่า MSO ยึดติดกับเนื้อขนมปัง ซึ่งการเติม MSO ร้อยละ 3 ไม่มีผลต่อคุณภาพของขนมปัง และการยอมรับของผู้บริโภค ในระหว่างการเก็บรักษาขนมปังเป็นเวลา 3 วัน พบว่าไม่มีการเปลี่ยนแปลงของปริมาตรขนมปัง และสีเปลือกของขนมปัง ส่วนขนมปังที่เติม MSO ร้อยละ 5 พบว่า เกิดปฏิกิริยาออกซิเดชันที่สูงกว่าขนมปังที่เติม MSO ร้อยละ 1 และ 3 ดังนั้น ขนมปังสามารถเสริมด้วย MSO ได้ถึงร้อยละ 3

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Major Program Food Science and Technology

Academic Year 2013

ABSTRACT

Lipids from cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) stored in ice for up to 6 days were extracted and characterized. The extraction yield of lipid from hepatopancreas was higher than those from cephalothorax. With increasing storage time, a progressive formation of hydroperoxide was evidenced by the increase in the absorbance band at 3600–3200 cm^{-1} in Fourier transform infrared (FTIR) spectra, and peroxide value (PV) ($p < 0.05$). The increases in thiobarbituric acid reactive substances (TBARS), ρ-anisidine value (AnV) and free fatty acid (FFA) content of lipids were noticeable when iced storage time increased ($p < 0.05$). Thus, the extended storage of raw material resulted in the enhanced deterioration of extracted lipids.

Impacts of extraction conditions on lipid and carotenoid yields from shrimp hepatopancreas were investigated. A mixture of hexane and isopropanol (50: 50, v/v) rendered lipid with the highest carotenoid yield (336.40 mg/kg hepatopancreas) with the extraction yield of 18.22% (w/w hepatopancreas) ($p < 0.05$). The use of hepatopancreas to solvent ratio of 1: 4.5 (w/v) and three repetitions showed the highest carotenoid yield (363.94 mg/kg hepatopancreas) with the extraction yield of 18.08% (w/w hepatopancreas). When astaxanthin (2 mg/g lipid) was added into lipid, the oxidation was lowered, in comparison with the control during the storage at 30 °C for 10 days as indicated by lower AnV. FTIR spectra study also revealed that triglyceride in lipid underwent oxidation to a lower degree when astaxanthin was incorporated.

Oxidative stability of lipid from shrimp hepatopancreas added with different essential oils at 200 ppm during 10 days of storage was studied. With increasing storage time, the progressive formation of hydroperoxide was found in all samples as evidenced by the increase in PV ($p < 0.05$). Among all essential oils

tested, lemon essential oil exhibited the highest preventive effect toward oxidation, followed by plai and basil essential oils, respectively. Nevertheless, the effectiveness for prevention of lipid oxidation of all three essential oils was slightly lower than α-tocopherol when used at the same level. The formation of volatile compounds and rancid odor of samples were lowered when being incorporated with essential oils. Thus, essential oils could be used as the potential natural antioxidant to retard oxidation and rancidity of lipid from hepatopancreas during extended storage.

Oxidative stability of shrimp oil-in-water emulsion (pH 3.6) incorporated with various antioxidants at different levels during the storage at 30 °C for 12 days was investigated. With increasing storage time, progressive formation of hydroperoxide was found in all samples as indicated by the increase in PV ($p < 0.05$). Sample added with 200 ppm α-tocopherol alone had the higher PV than others ($p < 0.05$). The increase in TBARS of all samples was noticeable when storage time increased ($p < 0.05$). This was concomitant with the decreased formation of volatile compounds and rancid off-odor. Emulsion containing mixed antioxidants, especially 50 ppm EDTA + 100 ppm tannic acid or 50 ppm EDTA + 100 ppm tannic acid + 500 ppm lecithin + 200 ppm α-tocopherol, had retarded the oxidation. Thus, the use of EDTA in combination with tannic acid could prevent the lipid oxidation in the shrimp oil-in-water emulsion more effectively, compared to other combinations of antioxidants tested.

Micro-encapsulation of shrimp oil using the mixture of whey protein concentrate (WPC) and sodium caseinate (SC) (1: 1, w/w) as a wall material was carried out. The impact of core/wall material ratios (1: 2 and 1: 4 (w/w)) and homogenizing pressures (2,000 and 4,000 psi) on characteristics and stability of emulsion was investigated. The size of emulsion oil droplets decreased with increasing homogenizing pressure ($p < 0.05$) but was not influenced by core/wall material ratio ($p > 0.05$). During the extended storage, particle size, flocculation factor (F_f) and coalescence index (C_i) of all emulsions sharply increased, especially in the emulsion prepared at 2,000 psi with a core/wall material ratio of 1: 2 ($p < 0.05$). After spray drying, micro-encapsulated shrimp oil (MSO) prepared at 2,000 psi with a core/wall material ratio of 1: 2 had the larger size than others ($p < 0.05$). MSO prepared using a core/wall material ratio of 1: 4 with homogenizing pressure of 4,000

psi exhibited higher encapsulation efficiency (EE) (51.3 - 52.8%) than others. Thus, both core/wall material ratio and homogezising pressure directly affected micro-encapsulation of shrimp oil.

The effect of different wall materials including WPC: SC (1:1, w/w), WPC: SC: gum arabic (1: 1: 2, w/w/w), WPC: SC: glucose syrup (1: 1: 2, w/w/w) and WPC: SC: maltodextrin (1: 1: 2, w/w/w) on micro-encapsulation was investigated. The highest EE was obtained when the mixture of WPC, SC and glucose syrup was used (84.43 – 88.19%), while the lowest EE was obtained for MSO using WPC and SC (50.37 – 53.05 %). Oxidative stability of MSO using the mixture of WPC, SC and glucose syrup as wall materials incorporated with different antioxidants was examined during storage at 30 °C for 6 weeks. With increasing storage time, the increase in PV was observed ($p < 0.05$). The lowest PV was found in those added with lemon essential oil + EDTA + tannic acid after 6 weeks of storage ($p < 0.05$). Thus, the use of appropriate wall materials in combination with selected antioxidants yielded MSO with high oxidative stability.

Characteristics and oxidative stability of bread fortified with MSO were determined. The addition of MSO could improve the loaf volume of breads. However, chewiness, gumminess and resilience of resulting bread were decreased. Bread crust and crumb showed higher redness and yellowness when MSO was incorporated ($p < 0.05$). Microstructure study revealed that MSO remained intact with bread crumbs. The addition of MSO up to 3% had no adverse effect on bread quality and sensory acceptability. During the storage up to 3 days, no changes in loaf volume and crust color were observed. Oxidation took place in bread fortified with 5% MSO to a higher extent, compared with those with 1 or 3% MSO. Therefore, the bread could be fortified with MSO up to 3%.

ACKNOWLEDGEMENT

I would like to express my special appreciation and sincere gratitude to my advisor Professor Dr. Soottawat Benjakul of the Department of Food Technology and my co-advisor Assoc. Prof. Dr. Aran H-Kittikun of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, who have shown the attitude and the substance of a genius in regard to researching and teaching. I would like to thank for their kindness, comments and helpful suggestion. Without their supervision and constant help, this dissertation would not have been possible.

I would especially like to thank my examining committee members, Asst. Prof. Dr. Manee Vittayanont and Dr. Nopparat Cheetangdee of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University and Assoc. Prof. Dr. Nurul Huda of the Food Technology Programme, School of Industrial Technology, Universiti Sains Malaysia, for the useful comments, remarks and engagement through the learning process of this Ph. D. thesis.

Furthermore, I would like to thank my parents for their endless love and support. Finally, I would also like to thank all of my dear friends for their most valuable help and friendship.

This study could not be succeeded without the financial support from National Research University Project of Thailand's Office of the Higher Education Commission and the Graduate School of and Prince of Songkla University through predoctoral scholarship.

Sirima Takeungwongtrakul

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CHAPTER 1
INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Thailand is one of major producer and exporter of shrimps. The crustacean processing industry generates an amount of by-products (mainly exoskeleton and cephalothorax), which could represent around 50-70% of the total weight of the raw material. Those by-products are the rich sources of biomolecules including lipid, carotenoid, chitin, proteins, flavors, nutritive components, and enzymes (Sila *et al.*, 2014). By-products are generally considered as low market value matter and mainly used as feeds or fertilizer. Additionally, the disposal and treatment of crustacean waste frequently require additional costs (Arancibia *et al.*, 2014). With improper disposal, they can cause the pollution. Hepatopancreas, removed from shrimp cephalothorax, is another value by-products generated from the manufacturing of whole shrimp excluding hepatopancreas. The hepatopancreas are rich in proteins, lipids and carotenoids. Lipids from crustacean have been paid increasing interest as the important source of n-3 fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have been known for health benefit (Treyvaud *et al.*, 2012). In addition, astaxanthin has been reported as the main carotenoid found in shrimp by-product. Astaxanthin was reported to be 10-fold stronger antioxidant activity than that of other carotenoids, namely zeaxanthin, lutein, canthaxanthin and carotene (Naguib, 2000) and 100-fold greater than that of α-tocopherol (Miki, 1991). Therefore, hepatopancreas can serve as the excellent source of carotenoid containing lipids, which can be further used as functional foods.

Recognition of the potential benefits of polyunsaturated fatty acids has stimulated the interest in fortification of foods with these lipids. Many lipids, especially from marine origin, are sensitive to heat, light, and oxygen and undergo oxidative damage very quickly. Lipid oxidation is a major cause of food deterioration and can affect the flavor, aroma, texture, shelf life, and color of food, which limits the use of lipid for food fortification (Ezhilarasi *et al.*, 2014; Lu *et al.*, 2013). To retard lipid oxidation, some synthetic antioxidants such as tert-butylhydroquinone (TBHQ),

butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), etc. are commonly incorporated in food systems. Nevertheless, the use of the synthetic antioxidants is limited or prohibited in many countries, especially in Europe and USA (Prior *et al.*, 2004). Therefore, the use of natural antioxidants is an alternative choice, which has gained increasing attention. In addition, the prevention of oxidative damage can be achieved by encapsulation of the oxidizable lipid, in which oxygen, trace metals, and other substances that attack its double bonds and other susceptible locations of fatty acid can be lowered (Damerau *et al.*, 2014; Josquin *et al.*, 2012).

However, a little information about lipids and carotenoids from white shrimp hepatopacreas, especially oxidative stability and their application, has been reported. The extraction of lipids from hepatopancreas of white shrimp can be a promising procedure to gain the high value added products from shrimp processing industry. Due to high susceptibility of lipid toward oxidation, the appropriate treatment, e.g. incorporation of antioxidant, encapsulation, etc. can lower the deterioration of lipids. As a consequence, it can be kept for an extended time and the quality can be maintained. Furthermore, it is of ease for application, particularly for supplementation in food products, e.g. bakery products, etc. The information gained can be of benefit for shrimp processing industry and pharmaceutical industry, in which the increasing revenue can be obtained and waste disposal can be reduced.

1.2 Review of Literature

1.2.1 Pacific white shrimp (*Litopenaeus vannamei*)

Pacific white shrimp, *Litopenaeus vannamei* (formerly *Penaeus vannamei*), is a marine crustacean belonging to the order Decapoda, a group of crustaceans that also includes lobsters and crabs. Pacific white shrimp are part of the Penaeidae family. The bodies of these animals are translucent but often have a bluish-green hue due to the presence of pigmented chromatophores (molecules evolved to collect/reflect light). The digestive tract of shrimp is divided into three main parts, the foregut, midgut and hindgut (Ceccaldi, 1989). The foregut (proventriculus, stomach) comprises the oesophagus and the part of the stomach where mastication occurs. The midgut gland or hepatopancreas (Figure 1) secretes digestive enzymes, absorbs

digested products and maintains mineral reserves (Ceccaldi, 1989). Hepatopancreas is an important gland for storage of nutrients such as lipids (Senphan and Benjakul, 2012), protein (Marsden *et al.*, 2007), carbohydrates (Zhou *et al.*, 2011), carotenoids (Senphan and Benjakul, 2012), vitamins (Nguyen *et al.*, 2012a), essential fatty acids and amino acids (Emerenciano *et al.*, 2013; Zhou *et al.*, 2011). The hindgut is a chitin-lined straight tube running from the cephalothorax dorsally through the abdomen to the rectum. Feed takes between 48.3 and 90.5 min to pass through the gut, irrespective of fibre, protein or lipid content. Some feed takes 4–6 h to pass through the gut (Ceccaldi, 1989).

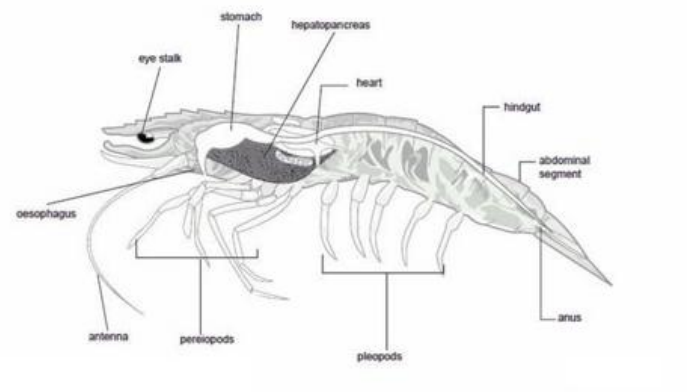

Figure 1 Shrimp digestive system
Source: FAO (2010)

According to Thai Customs, in January-March 2014, total shrimp exports of all shrimp product forms (not including freshwater prawns) reached 28,598 tons with the value of $369 million (Table 1). South Korea, Japan and the USA are the main importers of Thai shrimp (Table 2). The Food and Agricultural Organization of the United Nations (FAO 2010) reported that almost 90% of shrimp production in Thailand was farmed. Additionally, wild trawl-caught shrimp from Thailand are currently banned in the US. All of the shrimp products imported to the US from Thailand in recent years were farmed (Vietnam association of seafood exporters and producers).

Table 1 Thai shrimp exports in January-March 2014

Variety	January-March 2014	
	Volume (MT)	**Price (USD/kg)**
Frozen black tiger	497.4	13.65
Pacific white shrimp	10,890.4	12.95
Other frozen products	791.2	6.93
Black tiger products	624.8	8.48
Pacific white products	13,124.4	13.81
Other products	661.6	7.44
Canned black tiger	38.6	14.59
Canned Pacific white	1,756.0	12.15
Other canned products	213.9	12.61
Total	**28,598.4**	**12.92**

Source: Vietnam Association of Seafood Exporters and Producers (VASEP) website.

Table 2 Main destinations of Thai shrimp exports in January-March 2014

Variety	Destination	January-March 2014	
		Price (USD/kg)	Price (USD/kg)
Frozen black tiger	South Korea	123.1	13.32
Pacific white	USA	4,988.6	12.75
Other frozen products	Japan	344.2	6.15
Black tiger products	Japan	453.6	7.15
Pacific white products	USA	6,273.0	14.07
Other products	Japan	297.1	6.94
Canned black tiger	USA	21.6	17.34
Canned Pacific white	Japan	1,020.8	10.16
Other canned products	USA	116.8	5.59

Source: Vietnam Association of Seafood Exporters and Producers (VASEP) website.

1.2.2 Compositions of shrimp by-products

By-products from shrimp processing include cephalothorax, shell, etc. (Sachindra *et al.,* 2005). These by-products are economically recoverable, because they have high-quality protein, chitin, minerals, carotenoids, such as astaxanthin, and lipid that are high in n-3 fatty acids. Thus there is a great interest in developing alternative uses of those by-products to generate value-added products.

1.2.2.1 Protein and amino acids

Crustacean wastes are the important source of protein and amino acids. Protein contents of white shrimp head constituted 60.6% (dry basis) and essential amino acid content was 340.26 mg/g protein, indicating a high nutritional value for food and animal feed (Cao *et al.*, 2009). Bueno-Solano *et al*. (2009) produced liquid protein hydrolysates through lactic acid fermentation from shrimp (*Penaeus* spp.) by-products (heads and cephalothoraxes) and concentrated via vacuum evaporation at

80ºC or using a spray drying at 180 ºC/140 ºC (inlet/outlet temp). Protein content of dried hydrolysates ranged from 8.43 to 46.73 g/100 g of wet weight. The hydrolysates consisted of aspartic acid, glutamic acid, serine, glycine, histidine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine and phenylalanine. Mao *et al.* (2013) reported that free amino acid content in the fermented head shrimp waste was 2,061.79 mg/l. Essential α-amino acids accounted for 50.32% of total amino acids. Phenylalanine, lysine, and methionine were found at a high amount in the fermentation supernatant.

1.2.2.2 Chitin and chitosan

Chitin and chitosan are currently produced from several crustacean wastes. Chitin is a structural component in crustacean exoskeletons, constituting 15-20% chitin by dry weight (Coward-Kelly *et al.*, 2006). Manni *et al*. (2010) used a crude protease from *Bacillus cereus* SV1 for extraction of chitin from shell shrimp. Deproteinization of 88.8% was achieved using an enzyme/substrate ratio of 1: 20. The demineralization was completely performed within 6 h at room temperature in HCl 1.25 M, and the residual content of calcium in chitin was below 0.01%. Chitosan is a polymer of β-(1-4)-D-glucosamine units, which can be obtained by deacetylation of chitin, a polymer of β-(1-4)-N-acetyl-D-glucosamine (NAG) units (Bajaj *et al.*, 2011). The optimum condition for the deacetylation of shrimp wastes was observed at a temperature of 130 °C for 90 min, and the resulting chitosan had the MW of 150 kDa, and a deacetylation degree of 90% (Weska *et al.*, 2007). The effectiveness of chitosan in most applications depends on a complex mixture of electro-visco-elastic parameters. However, the high molecular weight of chitosan, which results in poor solubility at neutral pH and its high solution viscosity, limits its use in the food, cosmetics, agriculture and health industry (Xia *et al.*, 2011). Chitosan from shrimp wastes has been used as antitumour (Quan *et al.*, 2009), neuroprotective (Pangestuti and Kim, 2010), antifungal and antibacterial (Fernandes *et al.*, 2008) and anti-inflammatory (Yang *et al.*, 2010).

1.2.2.3 Carotenoid and carotenoproteins

Carotenoids are a group of fat-soluble pigments occurring widely in nature. The aquatic animals such as crustaceans are known to contain various carotenoids and are considered as one of the important sources of natural carotenoids (Matsumoto *et al.*, 2009). Yanar *et al*. (2012) reported that carotenoid contents of head, shell and whole body of shrimp (*Penaeus japonicas*, *Penaeus semisulcatus* and *Metapenaeus monoceros*) varied with seasons. Since crustaceans are incapable of synthesizing it or of converting it into vitamin A, and therefore it must be acquired from the diet (Hussein *et al.*, 2006). Astaxanthin (ASX), the main carotenoid found in shrimp by-product, is a red-orange carotenoid. It is mainly associated with the color of invertebrate animals. Sánchez-Camargo *et al*. (2011) identified various carotenoids by TLC in the redspotted shrimp (*Farfantepenaeus paulensis*) by-product (freeze-dried heads, shells and tails). Astaxanthin diester, astaxanthin monoester and free astaxanthin are the main pigments. ASX exhibited 10-fold higher antioxidant activity, compared with other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene and 100-fold greater than that of α-tocopherol (Miki, 1991). It is mainly used as a dyeing agent in the diets of aquaculture salmon and other species, but is also used in the cosmetic and pharmaceutical industries (Higuera-Ciapara *et al.*, 2006b). Generally, ASX occurs in crustaceans as carotenoproteins and carotenolipoproteins complexes (Higuera-Ciapara *et al.*, 2006a). Complexing of carotenoids to protein results in various colors displayed in crustaceans and provides stability to carotenoids, which are otherwise very unstable (Sowmya *et al.*, 2012).

Attempts have been made to recover carotenoids from shrimp waste either as carotenoids or as carotenprotein complex. Pigments from crustacean wastes can be extracted using vegetable lipids (Sindhu and Sherief, 2011). Several organic solvents have been permitted for use in food industries as carrier or extraction solvents. Some of organic solvents permitted for use in food industries are acetone, benzyl alcohol, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone, and ethanol, although levels of use depends on the type of foods in which they are used (FDA, 2010). A mixture of polar and non-polar solvents was reported to improve yields considerably, relative to single solvents (Sachindra *et al.*, 2006). Sachindra *et*

al. (2006) extracted carotenoids from shrimp waste using different organic solvents and solvent mixtures. A 50: 50 mixture of isopropyl alcohol and hexane gave the highest (43.9 µg/g waste) carotenoid extraction yield, compared to acetone, methanol, ethanol, isopropyl alcohol, ethyl acetate, ethyl methyl ketone, petroleum ether, and hexane individually and to a mixture of acetone and hexane. Mezzomo *et al.* (2011) found that the best solvents for carotenoid extraction from pink shrimp (*P. brasiliensis* and *P. paulensis*) residue (head and carapace) were acetone and hexane: isopropanol mixture. Sánchez-Camargo *et al.* (2012) used ethanol mixtures as cosolvent in supercritical CO_2 extraction of astaxanthin from Brazilian redspotted shrimp waste (*Farfantepenaeus paulensis*). Enzymatic hydrolysis of shrimp waste was found to enhance the oil extractability of carotenoids (Sachindra and Mahendrakar, 2011). The recovery of carotenoproteins from black tiger shrimp shells was maximized by the hydrolysis of shrimp shells using trypsin from bluefish (1.2 trypsin units/g shrimp shells) for 1 h at 25 ºC. Carotenoprotein contained 19.76% lipid and 87.91 mg total astaxanthin/g sample (Klomklao *et al.*, 2009).

1.2.2.4 Lipid and fatty acids

1.2.2.4.1 Compositions

Lipid is a major source of energy in marine invertebrates, including shrimp. It is involved in several essential processes for their growth, molting and reproduction (Zhang *et al.*, 2013). Lipid compositions of shrimp depend on many factors including sex, growth stage and season (Mili *et al.*, 2011). Lipid of shrimp head and shell from Indian white shrimp (*Penaeus indicus*) was extracted with the yield of 9.8% (dry weight basis) (Ravichandran *et al.*, 2009). Lipid can be extracted from krill, a shrimp-like crustacean (Sampalis *et al.*, 2003). Total lipid contents in Antarctic krill (*E. superba*) ranged from 15 to 30% (dry weight basis) and increased with maturity. *E. crystallorophias* had significantly higher lipid content than *E. superba* (Ju and Harvey, 2004). Krill oils contained predominantly phospholipids (20‑33%), polar non-phospholipids (64‑77%), and minor triglycerides (1‑3%) (Gigliotti *et al.*, 2011). Similar to fish lipid, krill and shrimp lipids are rich in long chain n-3 polyunsaturated fatty acids (PUFAs), mainly EPA (C20:5) and DHA (C22:6)

(Sánchez-Camargo *et al.*, 2011). Most of the marine fish have higher lipid content than the freshwater fish, whereas shrimp possesses the lowest lipid content (Li *et al.*, 2011). The marine fish and shrimps had much higher total n-3 PUFA than n-6 PUFA, while most of freshwater fish and shrimp demonstrated much lower total n-3 PUFA than n-6 PUFA (Li *et al.*, 2011). By-products (heads, shell and tail) of Northern shrimp processing were rich in n-3 PUFAs (7.8% EPA and 8.0% DHA) (Treyvaud Amiguet *et al.*, 2012). Fatty acids in the marine shrimp (*Penaeus brasiliensis*, *Penaeus schimitti* and *Xiphopenaeus kroyeri*) were C16:0, C20:5n-3, C22:6n-3, C18:1n-9, C18:0, C16:1n-7, C20:4n-6 and C18:1n-7. In the fresh water prawn (*Macrobrachium rosenbergii*), the major fatty acids were C16:0, C20:5n-3, C18:1n-9, C18:0, C22:6n-3, C18:2n-6, C17:0, and C18:1n-7 (Bragagnolo and Rodriguez-Amaya, 2001). Sánchez-Camargo *et al.* (2011) reported that EPA and DHA from the redspotted shrimp by-products (head, tail and shell) were 11.69% and 11.24% of the total fatty acids, respectively. In general, environmental and physiological factors affect the level of PUFAs in marine lipids (Eghtesadi-Araghi and Bastami, 2011). Crustaceans require dietary lipid as a source of essential fatty acids and other lipid classes such as phospholipids and sterols. Among all lipids, phospholipids are the predominant lipid class in crustacean (Nguyen *et al.*, 2012b). Lipid compositions of krill from the Scotia Sea and krill from the Gerlache Strait include phosphatidylcholine (PC) (33–36%), phosphatidylethanolamine (PE) (5–6%), triacylglycerol (TG) (33–40%), free fatty acids (FFA) (8–16%) and sterols (1.4–1.7%) (Fricke *et al.*, 1984). Thiansilakul *et al.* (2010) reported that the decreases in DHA and EPA contents suggested their susceptibility to oxidation during the extended storage. Intake of EPA and DHA may prevent inflammation, cardiovascular diseases (CVD) and reduce the symptoms in rheumatoid arthritis. Furthermore, PUFAs (n-3) play a role in preventing the promotion and progression stages of some types of cancer (D'Orazio *et al.*, 2012; Hu and Manson, 2012). PUFAs may have a positive effect in the treatment of depression and schizophrenia (Keller *et al.*, 2013; Meyer *et al.*, 2013). Additionally, DHA is essential for the development of brain, mammalian nervous system, eye development in fetus and infants. DHA is the predominant type of long-chain polyunsaturated fatty acids in the brain and represents around 15% of total fatty acids in that tissue. DHA in the phospholipids builds the structure of

neuronal membranes, while EPA may play a more important role in cardiovascular and immunological health (Ryan *et al.*, 2010).

1.2.2.4.2 Extraction of lipid

The recovery of lipid from shrimp by-product not only improves the economy value for shrimp processing industry, but also minimizes the environmental impact or pollution of this waste (Handayani *et al.*, 2008). In general, lipids occur in tissues in a variety of physical forms. The simple lipid is often part of large aggregates in storage tissues, from which is relatively easily extractable. On the other hand, the complex lipids are usually constituents of membranes, where they occur in a close association with proteins and polysaccharides. These lipids are not extracted readily. Generally, lipids are linked to other cellular components by hydrophobic, Van der Waal's forces, hydrogen bonds and ionic bonds (Xiao, 2010).

Lipid can be dissolved in a variety of solvents, depending on the relative strengths of the interactions between the solvent and either the hydrophobic or the hydrophilic regions of the molecules. Lipids with functional groups of low polarity, such as triacylglycerols or cholesterol esters, are very soluble in hydrocarbon solvents like hexane or cyclohexane, and in solvents of somewhat higher polarity, such as chloroform or ethers (Xiao, 2010). They tend to be rather insoluble in polar solvents such as alcohols, and methanol, etc. Solubility in polar solvents increases as the chain-length of the fatty acid moieties in these lipids decrease or as the chain-length of the solvent alcohol increases. Unsaturated lipid tends to dissolve in most solvents more readily than saturated and higher-melting analogues. In contrast, the polar complex lipid tend to be only sparingly soluble in hydrocarbon solvents, though dissolution can be aided by the presence of other lipids, but they dissolve readily in more polar solvents such as chloroform, methanol and ethanol (Xiao, 2010). In order to extract lipids from tissues, it is necessary to find solvents that will not only dissolve the lipids but will disrupt the interactions between the lipids and the tissue matrix (Xiao, 2010).

1.2.2.4.3 Methods for lipid extraction

Currently, a large number of methods are available to extract lipids from biological materials. Organic solvents, usually in mixtures containing chloroform and methanol have been widely used, such as the Bligh and Dyer (1959) and Folch *et al.* (1957) procedures. Automated extraction equipment, such as the Soxhlet or Goldfisch apparatus, has been successfully described, but its use requires long extraction times. Xiao *et al.* (2012) reoorted that soxhlet extraction with apolar solvents has low extraction efficiency for polar lipids, and the recovery of fatty acids in the extracts was below 50% in some samples. Acid hydrolysis causes the release of fatty acids bound in polar lipids, but there were still substantial amounts of fatty acids left in the residue (up to 17%). The Bligh and Dyer methodology seems to be slightly more efficient in releasing the polar lipids, but up to 10% of the fatty acids were left in the residue of some samples.

The Bligh and Dyer method is commonly used for determining polar lipid contents in environmental samples (Xiao, 2010). The Bligh and Dyer method is a simple adaptation of the Folch procedure and was developed merely as an economical means (in terms of solvent volumes) of extracting lipids from tissue, which contain relatively little lipid and a high proportion of water. The fat in the sample is extracted by a polar solvent mixture consisting of chloroform, methanol and water (1: 2: 0.8, v: v: v), which gives a one-phase system. After extraction, the one-phase system is separated into chloroform and methanol/water phases by addition of more chloroform and water. The lipids will follow the chloroform phase. The fat content is usually determined in an aliquot of the chloroform phase by weighing the lipids after evaporation of the solvent (Xiao, 2010). Bligh and Dyer method allows the extraction of all the lipids, including polar lipids, phospholipids and probably the lipids bound to other components of the cell membranes (Sánchez-Camargo *et al.*, 2011). Lipids from Brazilian redspotted shrimp waste were extracted with the yield of 3.3% and 4.9% (dry weight basis) by using hexane extraction and Bligh and Dyer method (chloroform: methanol: water), respectively. Hexane favors the extraction of only nonpolar compounds. Therefore, the yield was lower than that obtained using the Bligh and Dyer method (Sánchez-Camargo *et al.*, 2011). Mereakadeemia (2009)

extracted lipids from the edible part of *P. longirostris* and *P. maetia* by the Bligh and Dyer method. DHA, EPA and C20:4n-6 contents were 18.98%, 13.84% and 4.98% in the edible part of *P. longirostris* and were 415.59%, 12.84% and 3.34% in the edible part of *P. maetia.* Lipids in *P. vulgaris* (lobster), *N. norvegicus* (langoustine) and *P. kerathurus* (shrimp) muscles and cephalothorax were extracted by the Bligh and Dyer method with the yield of 0.70-1.30% and 1.30-2.40%, respectively (Tsape *et al.*, 2010). Gigliotti *et al.* (2011) reported that the highest extraction efficiency from Antarctic krill was achieved when one-step extraction (acetone: ethanol, 1: 1, v: v) with 1: 12 krill: solvent ratio (w: v) was applied, while Folch and Soxhlet methods rendered the lower yield. Extracted lipids contained predominantly phospholipids (20–33%), polar non-phospholipids (64–77%), and minor triglycerides (1–3%).

1.2.2.4.4 Methods for carotenoid containing lipid extraction

Many studies have been conducted to extract lipid and carotenoid, from shrimp by-product using various methods such as fermentation (Armenta and Guerrero-Legarreta, 2009), enzymatic (Klomklao *et al.*, 2009), supercritical (Mezzomo *et al.*, 2013), vegetable oils (Pu *et al.*, 2010) and organic solvent processes (Sánchez-Camargo *et al.*, 2011). A method has been patented for extraction of carotenoids from shrimp waste using a solvent mixture (Sachindra *et al.*, 2006). Several organic solvents have been permitted for use in food industries as carrier or extraction solvents. Some of the organic solvents permitted for use in food industries are acetone, benzyl alcohol, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone, and ethanol, although levels of use depends on the type of foods in which they are used (FDA, 2010). A mixture of polar and non-polar solvents was used to improve extraction yield of carotenoids from shrimp by-product (Khanafari *et al.*, 2007). Sachindra *et al.* (2006) reported the highest carotenoid yield (43.9 mg/kg hepatopancreas, wet weight basis) from head and carapace of *Penaeus indicus* when the carotenoid containing lipids were extracted with 50: 50 mixture of hexane and isopropanol. Mezzomo *et al*. (2011) reported that the best solvents for carotenoid extraction from head and carapace of pink shrimp were acetone and hexane: isopropanol mixture.

1.2.3 Lipid oxidation and antioxidants

Lipid oxidation of lipid and other PUFA-rich foods is a serious problem that leads to loss of shelf-life, consumer acceptability, functionality, nutritional value, and safety. PUFA oxidation affects the quality and nutritional value of foods (Aladedunye and Przybylski, 2009). The presences of fatty acid oxidation products in human foods, especially the aldehydes, have been implicated in ageing, mutagenesis, and carcinogenesis (Kampa *et al.*, 2007). The toxicity of these aldehydes such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal is due to their ability to crosslink proteins and bind covalently to nucleic acids (Nair *et al.*, 1986). Oxygen, light, heat, and irradiation accelerate lipid oxidation, thereby decreasing stability and shelf-life of products containing lipid. Due to low oxidative stability of polyunsaturated n-3 fatty acids, antioxidant is needed to use in lipid-enriched foods in order to prevent or reduce lipid oxidation (Sørensen *et al.*, 2010). Oxidation of lipid involves the reaction of unsaturated fatty acids with oxygen and occurs in 3 phases: an initiation or induction phase, a propagation phase, and a termination phase. The reactive products of the initiation phase react with lipid molecules, creating other reactive chemical products. This chain reaction, the continuation of further oxidation by propagation phase products, gives rise to the term 'autoxidation' (Kolanowski *et al.*, 2007).

The autoxidation (peroxidation) of lipid (lipid hydroperoxide) in homogeneous solution is a free radical chain reaction (Lagarde, 2010). This is a main mechanism for lipid oxidation, an autocatalytic process initiated by formation of radicals in unsaturated lipids followed by oxygen attack (Frankel *et al.*, 2002). Hydroperoxides are the primary oxidation products formed and further oxidation, decomposition and polymerization reactions lead to formation of a complex mixture of intermediate and secondary oxidation products such as aldehydes and ketones (Nawar, 1996). Non-volatile and volatile compounds with different molecular weight and polarity are formed (Dobarganes and Márquez-Ruiz, 2003).

1.2.3.1 Factors influencing lipid oxidation

Lipid oxidation in different systems can be varied and determined by different factors. Lipid oxidation in emulsions is generally recognized as being very complex as it may include oxidation or electron transfer events in all the different phases of the systems, making the mechanisms of lipid oxidation in emulsions very different and significantly more complex than lipid oxidation in bulk lipid systems. Although the basic oxidation reactions of lipids present in emulsions are exactly the same as those of lipids in bulk lipids, the factors affecting lipid oxidation in emulsions can be different. Intrinsic factors such as transition metal ions and pH, which affect lipid oxidation, differ significantly in emulsions and in bulk lipid systems (Jacobsen *et al.*, 2008).

1.2.3.1.1 Lipid composition

The rate of oxidation of free fatty acids (FFAs) increases as their degree of unsaturation increases. Thus, fats or oils that contain high concentrations of PUFAs are particularly prone to lipid oxidation. Generally, FFAs are more susceptible to autoxidation than esterified fatty acids in bulk lipids (Kinsella *et al.*, 1978). For water-in-oil emulsions, linolenic acid was most susceptible to oxidation, followed by linoleic and oleic acids, indicating that the oxidative capacity increased with increasing degree of unsaturation (Yi *et al.*, 2013). Alencar *et al.* (2010) showed that FFA concentrations of crude oil from soybean increased during storage. There are several factors which can impact FFA concentrations in crude oils, such as the initial FFA content, water content, and storage temperatures, oil type, etc. The presence of FFA can catalyze the further hydrolysis of TAG, thereby increasing the total FFA concentration in oil (Chen *et al.*, 2011). Mistry and Min (2006) found that FFAs had the ability to reduce surface tension of bulk soybean lipid as well as increased the diffusion rate of oxygen from the headspace into the lipid, thus increasing lipid oxidation rates. In o/w emulsions, FFAs are prooxidative due to their ability to concentrate at the emulsion droplet surface where they attract prooxidative transition metals that promote oxidation (Waraho *et al.*, 2009). The prooxidant activity of the FFAs was postulated to be due to their ability to attract prooxidant metals as well as

co-oxidize the triacylglycerol in the lipid (Waraho *et al.*, 2011). FFAs are surface active compounds because they are more polar than triacylglycerols due to the presence of unesterified carboxylic acid groups. The surface activity of FFAs allows them to diffuse and concentrate at the water-lipid interface of the o/w emulsions (Waraho *et al.*, 2009). Thus, FFAs could potentially make the emulsion droplet more negatively charged when pH values are above their pKa values (4.8-5.0 for medium- and long-chain ($C \geq 10$) fatty acids in aqueous solution) (Lieckfeldt *et al.*, 1995). Waraho *et al*. (2009) reported that addition of oleic acid (0-5.0% of lipid) to the emulsions increased lipid hydroperoxides and headspace hexanal formation. Negative charge of the emulsion droplet was also increased with increasing oleic acid concentration.

1.2.3.1.2 Pro-oxidants

Transition metal ions such as copper and iron commonly found in food emulsions are the major pro-oxidants that catalyze lipid oxidation. Copper has received somewhat less interest than iron because of its lower content in food, but it was reported to be as effective as or even more active in accelerating the decomposition of primary oxidation products (Yoshida and Niki, 1992). For each ion, the cuprous and ferrous are much more effective than the cupric and ferric ion in catalyzing oxidation (Wang and Wang, 2008). Transition metals originating in the aqueous phase are the most common cause of oxidative instability (Sun *et al.*, 2011). Transition metals are capable of directly breaking down unsaturated lipids into alkyl radicals, but this reaction occurs extremely slowly. The most likely mechanism for the acceleration of lipid oxidation in emulsions is the decomposition of lipid hydroperoxides (ROOH) into highly reactive peroxyl (ROO) and alkoxyl (RO) radicals by transition metals (Eq. 1 and 2) or other pro-oxidants. These radicals react with unsaturated lipids (LH) within the droplets or at the oil-water interface, which leads to the formation of lipid radicals (L° and LOO°) (Eq. 3 to 5). The lipid oxidation chain reaction propagates as these lipid radicals react with other lipids in their immediate vicinity (Eq. 6). Some of the lipid radicals formed may be terminated when they react with other radicals (Eq. 7) (McClements and Decker, 2006).

$Fe^{3+} + ROOH \longrightarrow Fe^{2+} + ROO^{o} + H+$ (Eq. 1)

$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{o} + OH^{-}$ (Eq. 2)

$ROO^{o} + LH \longrightarrow ROOH + L$ (Eq. 3)

$RO^{o} + LH \longrightarrow ROH + L$ (Eq. 4)

$L^{o} + O_2 \longrightarrow LOO^{o}$ (Eq. 5)

$LOO^{o} + LH \longrightarrow LOOH + L^{o}$ (Eq. 6)

$LOO^{o} + LOO^{o} \longrightarrow$ nonradical products (Eq. 7)

Formation of alkoxyl radicals (Eq. 2) also leads to β-scission reactions, that in turn result in the generation of a wide variety of different molecules, including aldehydes, ketones, alcohols, and hydrocarbons, which are responsible for the characteristic physicochemical and sensory properties of oxidized lipids. Lipid oxidation in o/w emulsions is highly dependent on the interfacial membrane of emulsion droplet, where prooxidants such as iron can interact with surface-active lipid hydroperoxides (McClements and Decker, 2006).

1.2.3.1.3 Multiphase system

Lipids in foods (such as milk, mayonnaise, salad dressings, margarine, soups, beverages, and desserts) are often present in the form of oil-in-water or water in-oil emulsions. Oxidation is believed to take place at a faster rate in the food emulsion systems because of the presence of multiple phases (Taneja and Singh, 2012). The possibility of oxidation taking place in the oil phase, in the aqueous phase, or at the interface makes the mechanisms very complex. The most important drivers of oxidation are metal ions, especially iron, which are present in trace amounts in most foods. Transition metals, such as iron, promote oxidation by preferentially residing at the interface and decomposing lipid hydroperoxides (Taneja and Singh, 2012). Factors affecting the rate of such reactions include the electrical charge on the droplet surface, the thickness of the interfacial layer, the presence of other components in the emulsion (such as protein and polysaccharides), types and

concentrations of antioxidants present, and dissolved oxygen concentration (Taneja and Singh, 2012). For bulk lipids, the oxidation reactions are mainly taking place at the interface between the oil and the air in the headspace above the oil or at the interface between the oil and air dissolved in the oil (Jacobsen *et al.*, 2008).

Overall oxidation rate is generally faster in emulsions than in bulk lipid, attributable to the large interfacial area in emulsions compared with the relatively small interface between air and lipid in bulk lipids. The total interfacial area in any emulsion obviously depends on the size distribution of the oil droplets (Mette *et al.*, 2007). Oxidation of lipid is the reaction between unsaturated lipid and oxygen. At low oxygen concentrations, it was observed that the rate-limiting step for lipid oxidation was the diffusion of oxygen through the aqueous phase. Under these oxygen limiting conditions, the rate of lipid oxidation increased with mechanical agitation and cooling because these processes increased the oxygen concentration. At high oxygen concentrations, oxygen diffusion was much faster than the rate of lipid oxidation, and so it was not limiting (Lu *et al.*, 2008). An effective means of retarding lipid oxidation is therefore to reduce the concentration of oxygen present; for example, by packing the food under vacuum or nitrogen (Naz *et al.*, 2004). Viuda-Martos *et al.* (2010) reported that the vacuum packaging of orange dietary fibre and oregano essential oil enriched bologna sausages showed the lowest lipid oxidation.

1.2.3.1.4 Emulsifier type and pH

The emulsifiers have been used to build the interfacial layer and stabilize emulsion. They can therefore be crucial for oxidative stability of emulsion. The type of emulsifier determines the structure and thickness of interfacial layer (Horn *et al.*, 2011) and emulsifiers can have different antioxidative properties (Haahr and Jacobsen, 2008). Protein-based emulsifiers may also increase the viscosity of the interfacial layer and the surrounding water phase, and thereby restricting the penetration and mobility of prooxidants into the lipid (Frankel *et al.*, 2002). Proteins represent GRAS food additives that can form physically stable o/w emulsions. Proteins can locate at the emulsion droplet interface in a manner that increases the oxidative stability of the lipid core (Hu *et al.*, 2003). Sodium caseinate and whey protein are important commercial milk protein used to stabilize emulsion in the food

industry (Ries *et al.*, 2010). Let *et al.* (2007) reported that peroxide value of the salad dressing with neat fish lipid or emulsified fish lipid (using whey protein) was similar, but at the end of the storage period, the PV of the salad dressing enriched with neat fish lipid was significantly higher than that in the salad dressing enriched with the emulsified fish lipid. Such an emulsion has successfully been incorporated into products such as yoghurt and meat products without imposing significant losses in sensory attributes of the final product (Chee *et al.*, 2005; Lee *et al.*, 2006). Whey proteins have been found to inhibit lipid oxidation in o/w emulsions when they are either at the emulsion droplet surface or in the aqueous phase. The antioxidant mechanisms of whey proteins have been attributed to their ability to form cationic charges on the surface of emulsion droplets, which repel transition metals; form thick viscoelastic films at emulsion droplet interfaces, which physically minimize lipid hydroperoxide-transition metal interactions; chelate prooxidative metals; and inactivate free radicals through their sulfhydryl groups and other amino acids (Tong *et al.*, 2006). Casein has been shown to be more effective antioxidant than whey protein. However, some reports found similar lipid oxidation levels in casein and whey protein containing emulsions. The level of oxidation was also dependent on total concentration of protein in the system (Ries *et al.*, 2010). The pH of emulsion system has been known to affect oxidative stability.

Charoen *et al*. (2011) found the oxidative stability of rice bran o/w emulsions depends on pH and the nature of the emulsifiers. Hu *et al.* (2003) showed that the rate of lipid oxidation in salmon oil-in-water emulsions stabilised with whey protein isolate, sweet whey, β-lactoglobulin or α-lactoglobulin was slower at pH 3 (proteins have positive charge) than at pH 7 (negative charge). This is due to the repulsive forces at pH 3 between the positively charged droplets and the positively charged prooxidants in the continuous phase. It was also reported that milk proteins have the ability to reduce the lipid oxidation rate when present in the emulsion continuous phase due to their ability to scavenge free radicals through their sulfhydryl groups (Elias *et al.*, 2005). Shahidi and Zhong (2011) reported that emulsifiers play a major role on activity of antioxidant by modifying the antioxidant distribution in the emulsified system. Emulsifier competes with antioxidants for localization at the

interface, or by self-arrangement of the emulsifiers which trap the antioxidants and carry them to the water phase to facilitate oxidation.

1.2.3.2 Antioxidants

Antioxidants are a group of chemicals effective in extending the shelf-life of a wide variety of food products. The use of antioxidants was extended to a wide variety of food products including high-fat foods, cereal and even products containing very low levels of lipids. The addition of antioxidants is one effective way to retard lipid oxidation. The most widely used antioxidants include free radical scavengers (also known as chain-breaking antioxidants) that inactivate free radicals formed in the initiation and propagation steps of lipid oxidation, and metal chelators (Shahidi and Zhong, 2011). Lipid peroxidation is a free-radical chain reaction that causes a total change in the sensory properties and nutritive value of food products (Rajapakse *et al.*, 2005). Decomposition of hydroperoxides by heating or by transition metal ion catalysis can produce both peroxyl and alkoxyl radicals. The formation of peroxyl radical is the major chain-propagation step in lipid peroxidation (Headlam and Davies, 2003; Niki, 1987). Antioxidants can break this chain reaction by reacting with peroxyl radicals to form unreactive radicals which are more stable or form nonradical products and that cannot propagate the chain reaction. In addition, it has been recognized that oxidative stress plays a significant role in a number of age specific diseases (Lu *et al.*, 2008). In bulk lipids, hydrophilic antioxidants locate preferentially at the oil-air interface and better protect the lipid from oxidation. In O/W emulsions, lipophilic antioxidants concentrate at the oil-water interface and inhibit lipid oxidation more effectively than hydrophilic antioxidants that partition into the water phase (Atarés *et al.*, 2012).

1.2.3.2.1 Classification of food antioxidants

Antioxidants counteract the oxidation in two different ways, by protecting target lipids from oxidation initiators or by stalling the propagation phase. Antioxidants can be broadly classified by the mechanism of action as primary

antioxidants (chain breaking antioxidants) and secondary antioxidants (preventive antioxidants) (Sun *et al.*, 2011).

1) Primary antioxidants

Primary antioxidants or chain-breaking antioxidants are free radical acceptors that delay or inhibit the initiation step or interrupt the propagation step of autoxidation through reaction with lipid and peroxy radicals and convert them to more stable, non-radical products (Gil, 2011). Primary antioxidants are most effective if they are added during the induction and initiation stages of oxidation when the propagation steps have not occurred. The most commonly used primary antioxidants in foods are synthetic compounds such as phenolic antioxidants including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tertiary butylhydroquinone (TBHQ) (Sun *et al.*, 2011). However, a few natural primary antioxidants such as tocopherols, flavonoids, as well as carotenoids are commonly added to foods (Sun *et al.*, 2011). The activity of phenolic antioxidants is often lost at high concentrations; therefore they may become prooxidative. This is due to their involvement in initiation (Sun *et al.*, 2011). Carotenoids belong to the most common auxiliary antioxidants. They are thought to be singlet oxygen quenchers and also react with chain-carrying peroxy radicals or alkyl-radical intermediates (Matsushita *et al.*, 2000).

2) Secondary antioxidants

Secondary antioxidants or preventive antioxidants act through numerous possible mechanisms other than converting free radicals to more stable products to slow the rate of oxidation. They can hinder reactive oxygen species (ROS) formation or scavenge species responsible for oxidation initiation (O^{-2}, $^1O_2$, etc.) (Sun *et al.*, 2011). There are many different preventive antioxidation pathways including chelation of transition metals, singlet oxygen deactivation, enzymatic ROS detoxification, UV filtration, inhibition of proxidant enzymes, antioxidant enzyme cofactors, etc. Moreover, they can replenish hydrogen to primary antioxidants, decompose hydroperoxides to nonradical species and act as oxygen scavengers, and

also act as reducing agent (Sun *et al.*, 2011). Because these secondary antioxidants can enhance the antioxidant activity of primary antioxidants, they are also called synergists. The commonly used secondary antioxidants are citric acid, ascorbic acid, ascorbyl palmitate, lecithin, and tartaric acid (Sun *et al.*, 2011).

1.2.3.2.2 Types of antioxidants

1) Synthetic antioxidants

Regarding lipid oxidation, additives such as sodium erythorbate, sodium ascorbate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ), propyl galate, nitrite (NO2) and nitrate (NO3) have been applied as antioxidants in foods containing lipids (Valencia *et al.*, 2007). However, the use of these synthetic antioxidants is negatively perceived by consumer due to potential toxicity and their connotation as chemicals in food (Georgantelis *et al.*, 2007). Synthetic antioxidants have varying activity, depending on the ability to scavenge radicals, etc. Sun and Ho (2005) tested various compounds using the DPPH• assay and found that BHT and TBHQ (0.1-1.0 mg/ml) had similar free radical scavenging activities. Conversely, Devi and Arumughan (2007) found that TBHQ had a higher free radical scavenging capacity than BHT.

2) Natural antioxidants

The demand for using natural antioxidants has increased due to the potential hazardous effects of synthetic antioxidants. Natural antioxidants have fascinated an immense deal of interest because of their health effects and positive image against degenerative diseases and certain cancers. Therefore, owing to consumer concerns about the safety of synthetic antioxidants, the declaration for natural antioxidants has been greater than before (Iqbal and Bhanger, 2007). In addition, a list of artificial compounds on food product labels seems incompatible with functional or organic claims. Based on this, food companies are trying to replace artificial additives with natural compounds, thus conferring a healthier image to their products (Botsoglou *et al.*, 2002).

2.1) Carotenoids

Carotenoids (Car) such as β-carotene, lycopene, zeaxanthin, lutein and canthaxanthin, etc. are found in some lipids, especially those from fish and crustacean (Opara and Al-Ani, 2010; Sánchez-Camargo *et al.*, 2011). They undergo no ultimate chemical change (physical quenching) (Foote and Denny, 1968). A process involving transfer of excited energy from $^1O_2$ to the carotenoids, resulting in the formation of ground state oxygen $^3O_2$ and triplet excited carotenoids (3Car*). The energy is dissipated through rotational and vibrational interactions between 3Car* and the solvent to recover the ground state of the carotenoids (Stahl and Sies, 1993). One molecule of β-carotene is estimated to quench up to 1000 molecules of singlet oxygen (Foote *et al.*, 1970). Sowmya and Sachindra (2012) reported that carotenoid extracts from shrimp processing showed strong antioxidant activity as indicated by radical scavenging, reducing activity and metal chelating activity, comparable to that of the known antioxidants α-tocopherol and TBHQ. Astaxanthin was found slightly better as an antioxidant in the emulsions than β-carotene. Both carotenoids had a marginal effect on iron-mediated oxidation in the emulsions. The main difference between two compounds is the presence of a keto-group and a hydroxyl group on the two β-ionone rings in the molecule of astaxanthin, which makes it more polar than β-carotene. This may have an influence on the location of the carotenoids in the emulsion (Kristinova *et al.*, 2014).

2.2) Tocopherols

Tocopherols, especially γ- and α-tocopherol, are the most abundant natural antioxidants in lipids (Wagner and Elmadfa, 2000). During autoxidation, they can act as chain breaking agent by scavenging free radicals and thus inhibiting initiation or stopping propagation. Tocopherols readily transfer an H-atom from their hydroxyl group on the chroman ring to lipid peroxyl radicals (LOO^o), leading to the formation of lipid hydroperoxides (LOOH) and tocopheroxyl radicals (TO^o). The tocopheroxyl radicals are resonance stabilized within the chromanol ring and therefore usually do not propagate the chain (Kamal-Eldin and Appelqvist, 1996). The optimum concentrations of α-, γ -, and δ-tocopherol to increase the oxidative stability

of lipid were 100, 250 to 500, and 500 to 1000 ppm, respectively (Evans *et al.*, 2002). In general, it has been reported that, under physiological conditions at around 37 ºC, the antioxidant activity is in the order α > β > γ > δ. The reverse trend δ > γ > β > α was observed at higher temperatures between 50 and 100 ºC (Khan and Shahidi, 2001). However, the antioxidant activity of tocopherols decreased and became prooxidant, depending on various factors, e.g. the methods of evaluation, the type of lipid, the degree of saturation, the temperature and the stage of oxidation, as well as on their concentrations (Jacobsen, 2010; Kim *et al.*, 2007). The presence of α-tocopherol in edible lipids is associated with a potential pro-oxidant activity, which was not observed for γ- and δ- tocopherol (Elisia *et al.*, 2013). Kim *et al.* (2007) reported the prooxidant mechanisms of oxidized α-tocopherol, which may be due to α-tocopherol peroxy radical, α-tocopherol oxy radical, hydroxyl radical,and singlet oxygen formed from the α-tocopherol. The oxidized α-tocopherol containing polar and nonpolar groups in the same molecule may reduce the surface tension of lipid to increase the transfer of headspace oxygen to lipid and accelerate the lipid oxidation (Kim *et al.*, 2007). Roman *et al*. (2013) reported the addition of α-tocopherol significantly delayed the formation of oxidation products, while chlorogenic acid did not enhance the oxidative stability of oils but protected α-tocopherol during heating.

2.3) Phospholipids

Phospholipids consist of a hydrophilic head group and a lipophilic tail group, in which the latter is extended into the emulsion oil droplet. Excess phospholipids that are not associated with the oil–water interface can also lead to the formation of micelles in the continuous phase (Horn *et al.*, 2011). Several mechanisms were proposed to account for the antioxidant activity of the phospholipids, including their ability to chelate metals, decompose lipid hydroperoxides and scavenge free radicals (Chen *et al.*, 2011). Besides the possible antioxidative properties of phospholipids in micelles, phospholipids at the interface might also possess antioxidant activity. However, the mechanisms by which phospholipids act are still under debate, possibly because the antioxidant mechanisms differ between different phospholipids classes (Wang and Wang, 2008). One mechanism that has been studied by several research groups is the ability of

phospholipids to work synergistically with tocopherols (Judde *et al.*, 2003). Some phospholipids, such as phosphatidylethanolamine, have also been proposed to possess antioxidant activity by their ability to interact with free radicals (Hamilton *et al.*, 1998). Furthermore, the successful use of phospholipids as antioxidants in lipids or emulsions has been shown to depend on the fatty acid composition of lipid and on the individual phospholipids used (Wang and Wang, 2008).

2.4) Ethylenediaminetetraacetic acid (EDTA)/citric acid

EDTA, a transition metal chelator, has been shown to be a very effective transition metal chelator, inhibiting lipid oxidation in oil-in-water emulsions (Klinkesorn *et al.*, 2005). The ability of EDTA to completely inhibit oxidation suggests that the transition metals naturally present in the oil and/or water enhance lipid oxidation (Cuvelier *et al.*, 2003). EDTA is able to hinder the lipid oxidation rate by preventing the transition metals from interacting with the hydroperoxide in the oil droplets. It has been demonstrated that EDTA reduces the rate of lipid oxidation in corn oil-in-water emulsions at both pH 3 and 7 conditions (Hu *et al.*, 2004). Xu *et al.* (2013) reported that emulsions containing EDTA at pH 4.0 and 7.0 exhibited significantly greater stabilities than those without EDTA after the storage. EDTA has been reported to be an inhibitor of lipid oxidation when the EDTA: iron ratio is greater than one. High concentrations of EDTA in relation to iron inhibit lipid oxidation by surrounding the metal and preventing interaction with peroxides (Mahoney and Graf, 1986).

Citric acid is another metal chelator commonly used in foods. Citric acid is found in almost all plant and animal species. It can chelate metal ions by forming bonds between the metal and the carboxyl or hydroxyl groups of the citric acid molecule. Citric acid is very effective in retarding the oxidative deterioration of lipids in foods and is commonly added to vegetable oils after deodorization (Hraš *et al.*, 2000).

2.5) Tannic acid

Tannic acid is composed of a central glucose molecule derivatized at its hydroxyl groups with one or more galloyl residues (Gülçin *et al.*, 2010).

Polyphenolic nature of tannic containing relatively hydrophobic ‘‘core’’ and hydrophilic ‘‘shell’’ are responsible for its antioxidant action (Isenburg *et al.*, 2006). Tannins also referred to as tannic acid, have a structure consisting of a central glucose and 10 galloyl groups. They are a type of water-soluble polyphenol that are present in the bark and fruits of many plants (Lopes *et al.*, 1999). Recently, Maqsood and Benjakul (2010a; 2010b) reported that tannic acid exhibited the superior radical scavenging activities as well as reducing power and effectively inhibited the lipid oxidation in fish mince, fish o/w emulsion and fish slices. Tannic acid is also affirmed as Generally Recognised As Safe (GRAS) by the Food and Drug Administration (FDA) at a level of 10–400 ppm for the use as an ingredient in some food products including meat products (Chung *et al.*, 2008).

2.6) Ascorbic acid

Ascorbic acid is an organic acid occurring widely in the vegetable world. Its antioxidant actions include the reaction with free-radical species or singlet oxygen quencher (Frankel *et al.*, 2002). Unfortunately, it has very low solubility in pure oils. Jayasinghe *et al*. (2013) revealed the pro-oxidant activity of ascorbic acid in such emulsions. Ascorbic acid is able to reduce Fe^{3+} to Fe^{2+}. Fe^{2+} decomposes lipid hydroperoxides quickly to form Fe^{3+} and lipid alkoxyl radicals. Compared to this reaction, the rate of decomposition of lipid hydroperoxide by Fe^{3+}, forming Fe^{2+} and a lipid peroxyl radical, is slower. Consequently, the reduction of Fe^{3+} by ascorbic acid accelerates lipid oxidation. The addition of ascorbic acid in concentrations between 40 and 800 ppm to fish oil enriched mayonnaises immediately promoted the formation of strong fishy, rancid, and metallic off-flavors. The intensity of these off-flavors increased with increasing levels of ascorbic acid (Jacobsen *et al.*, 2001). Ascorbyl palmitate, a lipid-soluble ester of vitamin C, is an approved synthetic antioxidant that delays the onset of rancidity (Beddows *et al.*, 2001). Lee *et al*. (1997) reported that ascorbyl palmitate can act as an effective oxygen scavenger in photosensitized oxidation reactions of vegetable oils.

2.7) Essential oils

Essential oils from various plants have been known to exhibit antioxidant property, which can extend shelf-life by lowering lipid oxidation in foods (Zivanovic *et al.*, 2006). The amount of essential oils used determines the acceptance of food products, as strong aroma of essential oils can be imparted to resulting products (Chouliara *et al.*, 2007). Essential oils can be extracted from several parts of plants, e.g. leaves, stems, roots, etc. Several plants contain varying essential oils. Basil contains high level of phenolic acids that contribute to its strong antioxidant capacity (Lee and Scagel, 2010). Özcan and Arslan (2011) reported that cinnamon and clove essential oils had higher antioxidant activity than rosemary essential oil in poppy and hazelnut oil. However, BHA was more effective than those essential oils. Essential oils were used for preparation of active films. Tongnuanchan *et al.* (2012) reported that film incorporated with lemon essential oil showed the highest ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP) ($p < 0.05$), while the other film incorporated with bergamot, kaffir lime, lemon and lime essential oils had lower activity.

1.2.3.3 Influence of antioxidants on lipid oxidation in bulk lipid and food emulsion

The effectiveness of antioxidants depends on several factors such as the polarity of the antioxidants, lipid substrate, pH, temperature, concentration of antioxidants, and the physical properties of the food (Huang *et al.*, 1997). In several studies, the ability of various antioxidants to inhibit lipid oxidation in bulk lipid and in o/w emulsions was compared. Polar antioxidants have properties described as the “antioxidant polar paradox”. A paradox is based on the hypothesis that polar antioxidants are more effective in bulk lipid and nonpolar antioxidants are more effective in o/w emulsions (Fang *et al.*, 2002). The antioxidant polar paradox is due to retention of nonpolar antioxidants in the lipid phase of o/w emulsions, or the ability of polar antioxidants to have a higher affinity toward the air-oil interface or reverse micelles, and thus they are able to concentrate on oil-air or oil-water interfaces where oxidative reactions would be the greatest (Sun *et al.*, 2011). An antioxidant added to

food must be effective at low concentrations (Sun *et al.*, 2011). A combination of antioxidants is used to obtain a synergistic effect. In a food product, a synergy is obtained by combining the added and inherent antioxidants. However, the behavior of antioxidants in food and their antioxidative ability can vary markedly, depending on the lipid-containing systems. Food products are predominantly multiphase systems.

1.2.3.3.1 Use of antioxidant in bulk lipid

One of the most effective ways of inhibiting lipid oxidation in bulk lipid is to incorporate antioxidants (Decker *et al.*, 2005). Tocopherols are the most common primary antioxidants present in many lipids, which may originate naturally from the extracted lipid itself or may be manually added after lipid refining (Frankel, 1996). However, tocopherols may not be the most effective antioxidants in bulk lipid systems. Hydrophilic antioxidants (Trolox or ascorbic acid) possess better antioxidant activity than their hydrophobic analogues (tocopherol and ascorbyl palmitate) in some bulk lipid systems (Huang *et al.*, 1996). The greater tendency for hydrophilic antioxidants to accumulate at the air–water interface where oxidation may be expected to begin is one of the mechanisms proposed to account for their better antioxidant activity in bulk lipid (Huang *et al.*, 1996). However, other mechanisms have also been proposed to account for the ability of hydrophilic antioxidants to act as better antioxidants than their hydrophobic analogs in bulk lipid since air is more hydrophobic than lipid and thus there is no driving force to concentrate hydrophilic antioxidants at the oil-air interface (Chaiyasit *et al.*, 2007). The addition of phospholipids to bulk lipid increased the antioxidant activity of tocopherol (Koga and Terao, 1995). It was postulated that the phospholipids formed microstructures, known as association colloids within the bulk lipid, which caused the tocopherol molecules to accumulate in the phospholipid microstructures where lipid oxidation primarily occurred. Ability of phospholipids to act as antioxidants was reported in various kinds of bulk lipids (Koga and Terao, 1995). Nevertheless, there is still a poor understanding of the importance and contribution of the combination of phospholipids and tocopherols to the oxidative stability of bulk lipids (Chen *et al.*, 2011). In bulk menhaden lipid, BHT was a more effective antioxidant than 4-hydroxymethyl-2,6-ditertiarybutylphenol, while α-tocopherol was more effective than δ-tocopherol

(Chaiyasit *et al.*, 2005). Sekhon-Loodu *et al.* (2013) found that polyphenols fractionated from frozen apple peel extract inhibited fish oil oxidation by 40‑62% at a total phenolic concentration of 200 µg/ml. The fractionated polyphenols from both dried and frozen apple peel showed higher inhibition of lipid oxidation, compared to α-tocopherol, butylated hydroxytoluene and crude apple peel extracts.

1.2.3.3.2 Use of antioxidant in food emulsion

The ability of a free radical scavenger to inhibit lipid oxidation depends on its chemical reactivity and physical location within an emulsion, e.g., oil, water and interfacial regions as shown in Figure 2. Non-polar antioxidants (α-tocophorol, ascorbyl palmitate, carnosol) are more effective antioxidants than their polar counterparts (trolox, ascorbic acid, carnosic acid and rosmarinic acid) since they accumulate at the oil-water interface where oxidation is prevalent (Chaiyasit *et al.*, 2007). Recently, antioxidants were esterified to lipophilic hydrocarbon chains to change polarity. Esterification of a hydrocarbon chain onto a free radical scavenger improved antioxidant activity in O/W emulsions such as ferulic acid (Hunneche *et al.*, 2008) and hydroxyltyrosol (Medina *et al.*, 2009). In real food systems, antioxidants can interact with other compounds in a manner that can either increase or decrease their reactivity (Alamed *et al.*, 2009).

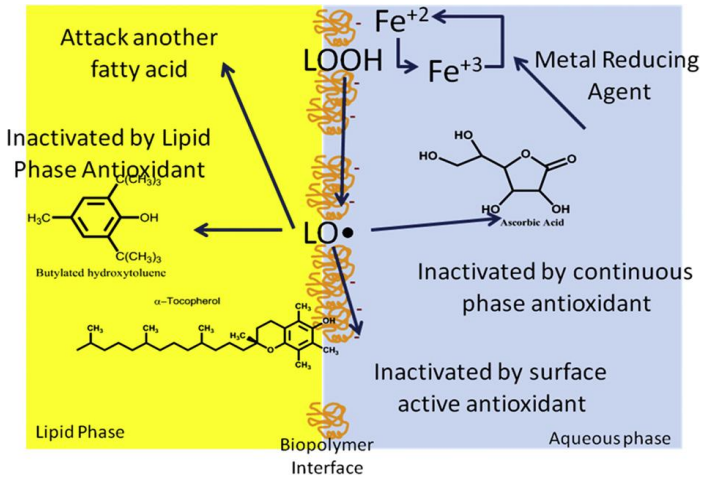

Figure 2 Potential reactions for lipid alkoxyl radical (LO^{o}) in an emulsions
Source: Waraho *et al.* (2011)

In O/W emulsions, chelating agents can inhibit lipid oxidation by decreasing metal reactivity or by physically partitioning the metal away from the lipid. Cho *et al.* (2006) found that chelating agents in the aqueous phase of an O/W emulsion could facilitate the transfer of iron out of the lipid droplets. EDTA has been shown to remove iron from the surface emulsions droplets. Overall, EDTA was found to be more effective at preventing lipid oxidation in o/w emulsions than sodium tripolyphosphate and citric acid (Hu *et al.*, 2004) and has been found to be effective in mayonnaise (Nielsen *et al.*, 2004) and in salad dressing prepared with fish lipid (Let *et al.*, 2007). Hu *et al.* (2004) indicated that the oxidative stability of whey protein isolate stabilized o/w emulsion could be increased by EDTA without having any impact on physical stability. Algae lipid emulsion stabilized by whey protein and protected by mixed tocopherols and EDTA was suggested as an oxidatively and physically stable n-3 PUFA delivery system (Djordjevic *et al.*, 2004). Emulsion stabilized by ascorbyl palmitate and rosemary extract, was shown to be relatively stable in surimi (Park *et al.*, 2004). Emulsions containing rosmarinate with 4 and 8 alkyl esters showed lower lipid hydroperoxides and headspace volatiles than those without rosmarinate and those with 0, 12, 18, and 20 alkyl esters. Phenolic free radical scavengers showed antioxidant capacities non-linearly in riboflavin photosensitized O/W emulsions. However, antagonistic rather than synergistic effects were observed in all rosmarinate alkyl esters with α-tocopherol. Rosmarinates with 4, 8, and 12 alkyl esters showed better antioxidant capacities than those with other alkyl chain length (Lee *et al.*, 2013). Dutch-style fermented sausages were manufactured with pork back-fat substitution by fish lipid or commercial encapsulated fish lipid, either added as such or as pre-emulsified mixture with soy protein isolate. Propanal and hexanal were much higher for sausages with fish lipid than for products with encapsulated lipid. Products with encapsulated or pre-emulsified lipid were significantly firmer than products from other treatments. Overall, it is technologically feasible to enrich dry fermented sausages with n-3 fatty acids from fish lipid and the application of commercial encapsulated fish lipid seems to be the best in retaining overall quality (Josquin *et al.*, 2012).

1.2.4 Encapsulation

Encapsulation may be defined as a process to entrap one substance within another substance. The encapsulated substance can be called the core, fill, active, internal or payload phase. The substance used for encapsulating the target substance is often called the wall, coating, membrane, shell, capsule, carrier material, external phase, or matrix (Fang and Bhandari, 2010; Wandrey *et al.*, 2010a). Encapsulation is a useful tool to improve delivery of bioactive molecules (e.g. antioxidants, minerals, vitamins, lutein, fatty acids, lycopene) and living cells (e.g. probiotics) into foods (De Vos *et al.*, 2010; Wandrey *et al.*, 2010b). Encapsulation aims to enhance the stability of bioactive compounds during processing and storage and to prevent undesirable interactions with food matrix. Mainly, bioactive food compounds can undergo degradation or deterioration with ease. These compounds would profit from an encapsulation procedure, since it slows down the degradation processes (e.g., oxidation or hydrolysis) or prevents degradation until the product is delivered at the desired sites (Lesmes and McClements, 2009).

1.2.4.1 Spray-drying

Microencapsulation by spray-drying has been successfully used in the food industry for several decades, and this process is one of the oldest encapsulation methods (Gouin, 2004). Spray-drying has been used to encapsulate active material within a protective matrix formed from a polymer or melt (Gharsallaoui *et al.*, 2007). Although many techniques have been developed to microencapsulate food ingredients, spray-drying is the most common technology used in food industry for the preparation of dry stable additives such as essential lipids and flavors due to low cost and available equipment (Kha *et al.*, 2010). It results in powders with good quality, low water activity, easier handling and storage and also protects the active material against undesirable reactions. Compared to freeze-drying, the cost of spray-drying method is 30–50 times cheaper (Desobry *et al.*, 1997).

1) Principle of spray-drying

Spray drying, or drying by atomization, refers to the removal of moisture from fluid material (solution, dispersion or paste) by breaking it into small droplets in the presence of hot air to obtain a dry powder. In the spray-drying process, the liquid feed is pumped into the drying chamber through an atomizing system (Al-Asheh *et al.*, 2003). Spray-drying produces a very fine powder (10–50 μm) or large size particles (2–3 mm). Microencapsulation using spray-drying process involves three basic steps (Dziezak, 1988).

1.1) Preparation of the dispersion or emulsion

1.2) Homogenization of the dispersion

1.3) Atomization of the mass into the drying chamber

The first stage is the formation of a fine and stable emulsion of the core material in the wall solution. The mixture to be atomized is prepared by dispersing the core material, which is usually of hydrophobic nature, into a solution of the coating agent, which is immiscible. The dispersion must be heated and homogenized, with or without the addition of an emulsifier, depending on the emulsifying properties of the coating materials because some of them exhibit interfacial activities (Gharsallaoui *et al.*, 2007). Oil droplets should be rather small and viscosity should be low enough to prevent air inclusion in the particle (Drusch, 2007). The core material retention during microencapsulation by spray-drying is affected by the composition and the properties of the emulsion and by the drying condition (Jafari *et al.*, 2008b). The obtained o/w emulsion is then atomized into a heated air stream supplied to the drying chamber and the evaporation of the solvent, usually water, consequently leads to the formation of microcapsules. As the sprayed particles fall through the gaseous medium, they assume to have a spherical shape with the lipid encased in the aqueous phase (Gharsallaoui *et al.*, 2007).

2) Preparation of emulsion

Emulsification is one of the important and critical steps in micro-encapsulation of food lipids through spray drying and emulsion properties such as stability and droplet size plays a key role in optimizing the encapsulation efficiency during the process (Barbosa *et al.*, 2005). A stable emulsion with minimum droplet size can extend the shelf-life of encapsulated oil products through reduction of unencapsulated oil at the surface of powder particles (Jafari *et al.*, 2007). Based on emulsion droplet size, emulsions can be divided into micro- (10–100 nm), mini (nano)- (100–1000 nm) and macro-emulsions (0.5–100 μm) (Windhab *et al.*, 2005). Jafari *et al*. (2008a) studied characteristics of the spray dried powders from oil-whey protein emulsions with three different emulsification methods including Silverson (rotor-stator system), ultrasound and Microfluidizer. Powders produced from Microfluidized emulsions provided the minimum surface oil coverage (18%), followed by ultrasound (20%) and Silverson (rotor-stator system) (35%), respectively. The morphology of the obtained powers was varied.

High-pressure homogenization is well known to play a vital role in emulsification process. Diagram of high pressure homogenizer is illustrated in Figure 3. The main features of a microfluidizer consist of an air-driven intensifier pump and a fixed-geometry interaction chamber. The intensifier pump provides high pressure to force the crude emulsion through the interaction chamber of the microfluidizer. The crude liquid emulsion can be divided into two process streams. It enters the two well defined microchannels and the emulsions are dramatically accelerated to a very high velocity as they pass through the chamber. Two jets of the liquid emulsions then collide with one another. Forcing the flow stream by high pressure through microchannels toward an impingement area creates a tremendous shearing action which can produce an exceptionally fine emulsion (Tang *et al.*, 2013). This technique also exploits the highly turbulent shear force induced by high pressure along with cavitation to rupture the emulsion into exceptionally fine droplets (Olson *et al.*, 2004).

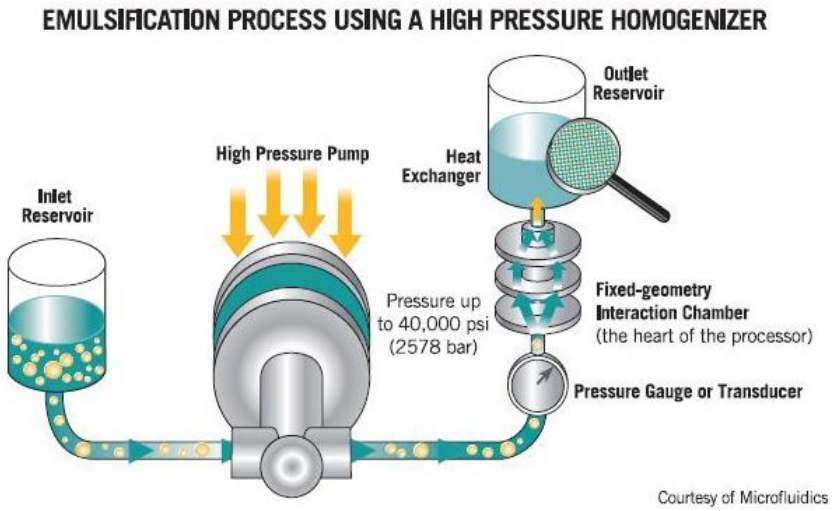

Figure 1 Schematic diagram of Microfluidizer

Source: Particle Sciences Drug and Development Service (2009)

High-homogenization pressure is widely used to emulsify, disperse, homogenize and to reduce droplets size, in order to obtain emulsions stables to coalescence (Kaushik and Roos, 2007). The mean particle diameter of the emulsion decreased with increasing homogenization pressure and number of passes. When particle size was decreased, the attractive forces acting between the droplets were reduced. Thus the better emulsion stability against droplet flocculation and coalescence can be achieved (Qian and McClements, 2011). It is also expected to improve the coating ability due to increased surface activity of the emulsifying molecules (Shukat and Relkin, 2011). When emulsifiers get associated on newly formed oil droplet surface, a protective membrane which prevents oil droplets from coalescence can be formed. Due to their small droplet size (from 100 to 500 nm) and narrow particle size distributions, nano-emulsions have been shown to present a long-term stability against creaming or sedimentation (Relkin *et al.*, 2009; Relkin *et al.*, 2008; Solè *et al.*, 2006). Carmona *et al.* (2013) reported that homogenizing pressures below 500 bar showed the increase in pressure, which led to a reduction on droplet size. On the other hand, the increase in the homogenization pressure resulted in higher droplets size. This phenomenon in which the droplet size increases with increasing energy emulsification is termed as over-processing and can be attributed to the

underperformance of emulsifiers and to an increase in the droplets Brownian motion, resulting in higher probability of collision and coalescence (Mahdi Jafari *et al.*, 2006). The increase in interfacial area during homogenization can be another reason. When interfacial area becomes too large, the wall materials (emulsifiers) can no longer sufficiently cover the droplets (Huynh *et al.*, 2008). Additionally, the increase of homogenization pressure led to a reduction of encapsulation efficiency. This result is related to the emulsion droplet size. According to Jafari *et al.* (2008b), the higher surface oil in the particles produced from emulsions with larger droplets can be attributed to the droplets breakdown during atomization, resulting in lower encapsulation efficiency.

3) Operating conditions of spray-drying

In order to obtain good microencapsulation efficiency, the wall material must be suitable and the optimal spray-drying conditions must be used. The main factors in spray-drying that must be optimized are feed temperature, air inlet temperature and air outlet temperature (Liu *et al.*, 2004).

3.1) Feed temperature

Feed temperature modifies the viscosity and fluidity of the emulsion, thus determining its capacity to be homogenously sprayed. When the feed temperature is increased, viscosity and droplets size should be decreased but high temperatures can cause volatilization or degradation of some heat sensitive ingredients. The rate of feed delivered to the atomizer is adjusted to ensure that each sprayed droplet reaches the desired drying level before it comes in contact with the surface of the drying chamber (Zbicinski *et al.*, 2002). Emulsion viscosity and particle size distribution have significant effects on microencapsulation by spray-drying. High viscosities interfere with the atomization process and lead to the formation of elongated and large droplets that adversely affect the drying rate (Wan *et al.*, 2011).

3.2) Air inlet temperature

Air inlet temperature is directly proportional to the microcapsule drying rate and the final water content. When the air inlet temperature is low, the low evaporation rate causes the formation of microcapsules with high density membranes, high water content, poor fluidity, and easiness of agglomeration. However, a high air inlet temperature causes an excessive evaporation and results in cracks in the membrane inducing subsequent premature release and a degradation of encapsulated ingredient or also a loss of volatiles (Gharsallaoui *et al.*, 2007). The air inlet temperature is usually determined by two factors: the temperature which can safely be used without damaging the product or creating operating hazards. The comparative cost of heat sources must be considered (Zakarian and King, 1982). Chen *et al.* (2013) studied the effect of air inlet temperatures (160, 170 and 180 °C) and air outlet temperatures (70 and 80 °C) on the characteristic of fish oil encapsulated by the mixture of whey protein isolate and caseinate (ratio of 4: 1). At the same outlet temperature, a higher inlet temperature decreased the microencapsulation efficiency of microcapsules. The inlet temperature of 170 °C and outlet temperature of 70 °C exhibited the highest microencapsulation efficiency (48.9%) (Chen *et al.*, 2013).

3.3) Air outlet temperature

Air outlet temperature, obtained under given conditions, can be considered as the control index of the dryer. It is quite difficult to predict this outlet temperature in advance for a given product, since it depends on the drying characteristics of the material (Gharsallaoui *et al.*, 2007). Contrary to the air inlet temperature, the air outlet one cannot be directly controlled since it depends on the air inlet temperature, and the ideal air outlet temperature for the microencapsulation of food ingredients such as flavors has been reported to be 50–90 ºC. Chen *et al.* (2013) reported that microencapsulation efficiency of encapsulated fish oils could be enhanced by drying at the optimum air outlet temperature (70 °C). This optimum condition also rendered smaller reconstituted particle size (0.35 μm) when compared to those prepared under air outlet temperature of 80 °C (1.0 μm). The lower microencapsulation efficiency and large reconstituted particle size might be due the

extent of protein denaturation and aggregation, influenced by the higher outlet temperature. The best spray-drying conditions are compromise between high air temperature, high solid concentration of the solution, and easy pulverization and drying without expansion and cracks of final particles (Gharsallaoui *et al.*, 2007).

Microencapsulation efficiency can be increased by increasing wall solid concentration which can be related to the formation of surface core prior to the formation of crust around the drying droplets (Young *et al.*, 1993). Kha *et al*. (2010) found that a good quality Gac powder in terms of color, total carotenoid content and total antioxidant activity can be produced by spray-drying at inlet temperature of 120 ºC and adding maltodextrin concentration at 10% w/v. The main limitation of the spray-drying technique in microencapsulation is the limited number of wall materials available and that must have a good solubility in water (Fuchs *et al.*, 2006). Barley protein-stabilized fish lipid microcapsules (1–5 μm) were successfully prepared by a pre-emulsifying process. The optimal conditions for microcapsule formation were 15% protein and a 1.0 oil/protein ratio. Microcapsules could be converted into free-flowing powders by a spray-drying process at an optimum inlet temperature of 150 °C. These microcapsules exhibited high lipid encapsulation efficiency, loading efficiency, and low moisture content (Wang *et al.*, 2011).

3.4) Materials used for encapsulation

The selection of wall material influences the emulsion stability during its formation and upon the drying process and also determines the characteristics of resulting microcapsules (Ré, 1998). Materials used as protective shell of encapsulates must be food-grade, biodegradable and able to form a barrier between the internal phase and its surroundings. Many proteins have been used for stabilizing emulsion prior to encapsulation of fat based products. Proteins used for microencapsulation of oils and fats include whey protein (Partanen *et al.*, 2008) and caseinate (Drusch *et al.*, 2012; Pan *et al.*, 2013). Increasing degree of casein aggregation in spray-dried homogenized emulsion of fish lipid was shown to improve the stability of lipid toward oxidation (Keogh *et al.*, 2001).

Protein-carbohydrate conjugates are stable over a greater range of pH compared with the unmodified protein, making them more suitable for use in a wider

range of foods. The use of a combination of proteins and carbohydrates as encapsulants has been found to be excellent for microencapsulation. The incorporation of carbohydrates has been shown to improve the drying properties (Drusch *et al.*, 2007; O'Regan and Mulvihill, 2010). Incorporation of dextrin with proteins as encapsulant material for fish lipid powders increased the oxidative stability of lipid (Kagami *et al.*, 2003). Hogan *et al.* (2001) found that the microencapsulation efficiency of lipid increased with increasing dextrose equivalent of the carbohydrate that was used in combination with sodium caseinate. Jayasundera *et al.* (2009) found that the combination of sodium caseinate with lactose could reduce the shrinkage of microcapsule. Lactose can replace the water to some extent and keep the protein solubilized after drying. This leads to the increase in stability of the sodium caseinate film on the powder surface, and less fat leaks out onto the powder surface. Tonon *et al.* (2011) reported that maltodextrin in combination with whey protein concentrate were the wall material that protected the active material against lipid oxidation most effectively. Maltodextrin is considered as a suitable drying aid to preserve its color and antioxidant properties. It has been used in the micro-encapsulation of food components. High dextrose equivalent maltodextrin protected the encapsulated orange peel lipid against oxidation, suggesting the importance of dextrose equivalent to the functionality of the wall system (Kagami *et al.*, 2003). Drusch *et al*. (2007) emphasized that glucose syrup with a high dextrose equivalent is less permeable to oxygen and offers better protection to encapsulated flavors, compared to carbohydrates with a low dextrose equivalent value. Furthermore, it has been shown that the addition of mono- and disaccharides to maltodextrins reduces pore size in the maltodextrin network and limits oxygen diffusion, emphasizing that not only the dextrose equivalent of a maltodextrin or glucose syrup but also its molecular weight distribution determines the stability of the encapsulated core material.

The skim milk powder, 70% skim milk powder + 30% maltodextrin, 70% skim milk powder + 30% lactose, 70% skim milk powder + 30% sucrose, and all of these wall materials were used for emulsion preparation. The emulsions were dried in mini-spray dryer at drying air temperature of 175°C. The microcapsules produced by 70% skim milk powder + 30% lactose and 70% skim milk powder + 30% sucrose

were selected as the best microcapsules according to high encapsulation efficiency and acceptable level of peroxide value (Aghbashlo *et al.*, 2012a). In addition, protein-carbohydrate conjugates formed by the Maillard reaction have good emulsifying and antioxidant properties (Kato, 2002). The antioxidative properties of Maillard reaction products offer protection to unsaturated lipids (Dong *et al.*, 2011). Augustin *et al.* (2006) confirmed that Maillard reaction products obtained by heating a mixture of protein sources and carbohydrates had a positive influence on encapsulation efficiency. Additionally, the ratio of core to wall material also affects the quality of microencapsulated oil. Aghbashlo *et al.* (2012b) studied the effect of oil concentration (10-30%, based on total solids) and water content (70-90%) on encapsulation efficiency when skim milk protein was used as wall material for fish oil microcapsulation. Increasing oil proportion negatively affected the encapsulation efficiency when the same water content was used. This was attributed to insufficient amount of wall material. As a consequence, wall material could not completely cover the oil droplets at the higher oil proportion (Zhong *et al.*, 2009).

1.2.4.2 Influence of encapsulation and antioxidant on lipid oxidation

Efficient combinations of antioxidants for the stabilization of bulk fish lipid high in γ- or δ-tocopherol and low α-tocopherol in combination with lecithin or citric acid esters from monoglycerides (Citrem) and ascorbyl palmitate were reported (Drusch *et al.*, 2008). Olive lipid in the absence or presence of 300 ppm caffeic acid was encapsulated using 1.5% w/w sodium alginate. Encapsulated olive lipid with and without added caffeic acid and unencapsulated olive lipid were stored at 20 or 37 ºC for 30 days. It was found that caffeic acid addition increased the stability of the final lipid product. Oxidation was generally slower in the encapsulated lipid samples. Both encapsulation and addition of caffeic acid preserved unsaturated fatty acids (Sun-Waterhouse *et al.*, 2011). Baik *et al.* (2004) reported that encapsulated fish lipid was 10 times more stable against oxidation than the surface fish lipid. Tocopherol, which is a lipophilic antioxidant, showed a greater antioxidative effect in both surface and encapsulated fish lipid than ascorbyl palmitate, which is an amphiphilic antioxidant. In the microencapsulated fish lipid, the addition of rosemary extracts rich in carnosic

acid to ternary blends of tocopherols, ascorbyl palmitate and lecithin or Citrem significantly retarded autoxidation (Serfert *et al.*, 2009).

1.2.5 Supplementation of micro-encapsulated oils in bakery products

Currently, functional foods have been dominating the food market (Kadam and Prabhasankar, 2010). Foods enriched with n-3 PUFA, may be classified as functional foods, by acting on human health (De Conto *et al.*, 2012). The greatest difficulty for the fortification of food with oil containing EPA and DHA is due to high unstability and susceptibility to oxidation in the presence of light and oxygen, loss in their functional and sensory qualities (Arab-Tehrany *et al.*, 2012). In order to be incorporated into food formulations, a maximum limit should be observed to avoid affecting sensory acceptance (De Conto *et al.*, 2012). Microencapsulation is one of the strategies used by industry to protect the PUFAs of external factors, which initiate the oxidation process. Those reactions can produce off-flavors, both during processing and storage. Moreover, micro-encapsulation also mask any unwanted odor and flavor in the final product and to facilitate handling (Kolanowski and Laufenberg, 2006). Bakery products are an excellent product in which incorporation of 'functional foods' is attempted. The addition of n-3 PUFA could improve essential fatty acid intake. Therefore, the use of bakery as vehicles for different micronutrients has been extended (Kadam and Prabhasankar, 2010).

Bakery making is a complex process mainly consists of mixing, fermentation and baking, during which water evaporation, volume expansion, starch gelatinization; protein denaturation and crust formation occur (Sivam *et al.*, 2010). So, The addition of any extra compounds can influence the baking process and results in qualitative changes in the bakery (Ezhilarasi *et al.*, 2013). Borneo *et al.* (2007) added encapsulated n-3 fatty acids in cream-filled sandwich cookies without any adverse effect on sensory properties. However, the addition of micro-encapsulated n-3 fatty acids affected the characteristics (specific volume, firmness, L* and C*) and sensory characteristics (appearance, aroma and overall acceptance) of white pan breads (De Conto *et al.*, 2012). Bread with whey protein isolate (WPI) encapsulates exhibited higher volume, softer crumb texture, desirable color and sensory attributes and had higher free (-)-hydroxycitric acid concentration. This indicated that WPI had

an excellent encapsulation efficiency than maltodextrin (MD) and combination of WPI and MD (1:1 ratio) during bread baking (Ezhilarasi *et al.*, 2013). Gökmen *et al.* (2011) and Ezhilarasi *et al*. (2014) reported that increasing amount of micro-encapsulated oil or active compounds decreased loaf volume of bread. Moreover, encapsulation was also effective in masking of the flavor of phenolic compounds and increasing the resistance of phenolic compounds to the thermal effects when capsules were incorporated into cakes (Luca *et al.*, 2014). Gökmen *et al*. (2011) developed functional bread enriched with encapsulated flax seed oil. Encapsulation decreased lipid oxidation in breads during baking. The formation of nonanal and hexanal was more pronounced in bread containing flax seed oil in the free form than in the encapsulated form. Thus, bread should be fortified with n-3 PUFA in the form of microencapsulated powder. Bread containing micro-encapsulated oil encountered 11–20% losses. However, no additional loss occurred during the storage. From the sensory evaluation, bread containing 1.0% microencapsulated oil was acceptable. In bread with a 2.5% inclusion of microencapsulated oil, the fishy flavor increased while the palatability, especially after the storage, decreased (Henna Lu and Norziah, 2011).

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1.4 Objectives

1.3.1 To investigate the changes of lipids and carotenoid in lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp.

1.3.2 To study the extraction conditions for carotenoid containing lipids from hepatopancreas of Pacific white shrimp and the role of astaxanthin in prevention of oxidaton in lipid extracted from hepatopancreas during extend storage.

1.3.3 To elucidate the effect of different essential oils on lipid oxidation of lipids from hepatopancreas of Pacific white shrimp during storage.

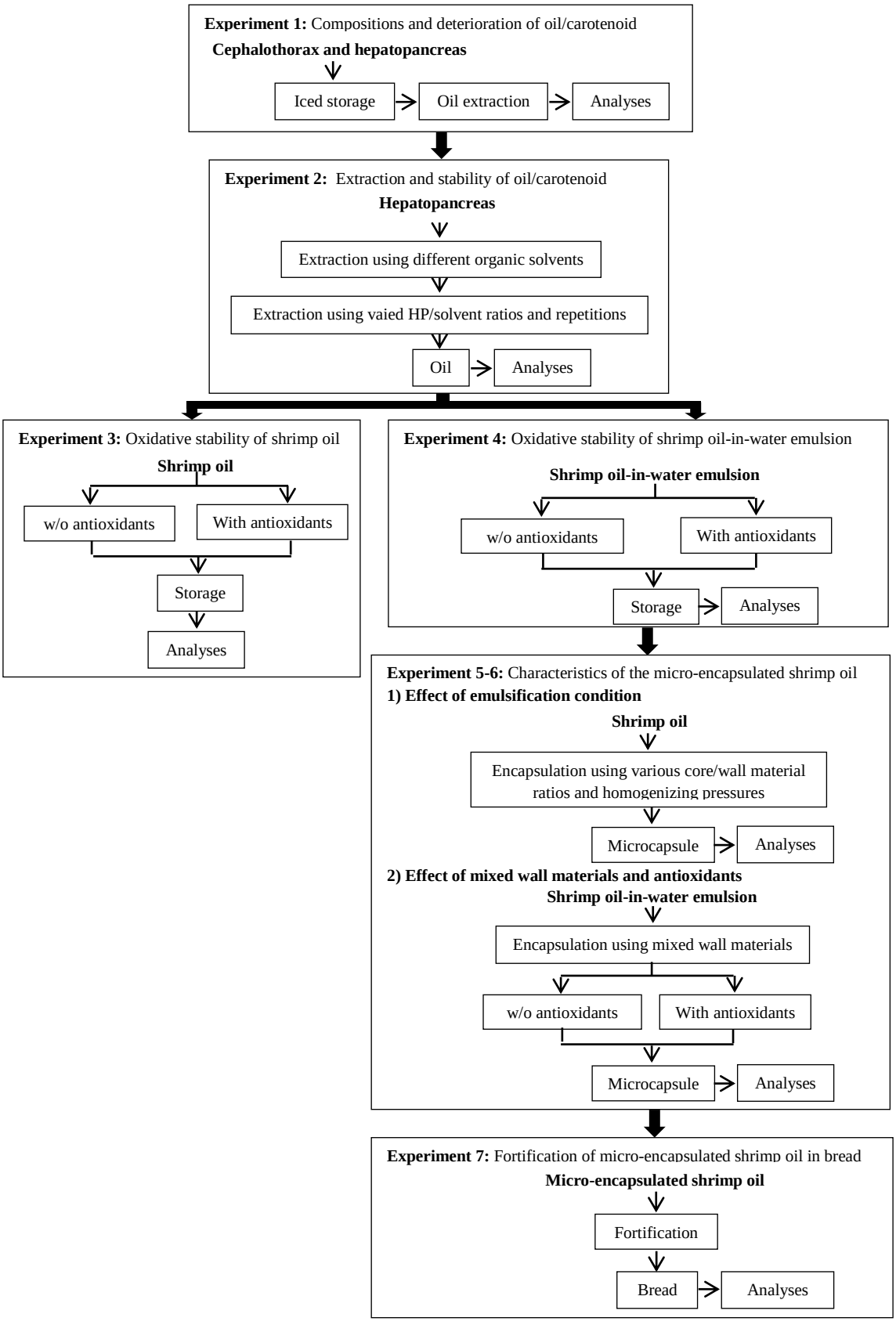
1.3.4 To investigate the oxidative stability and rancidity of shrimp oil-in-water emulsion incorporated without and with various antioxidants during storage.

1.3.5 To investigate the impact of homogenization at varying pressure levels and the ratio of core/wall material on characteristics of encapsulated shrimp oil.

1.3.6 To study the influence of mixed wall materials on encapsulation effeiciency and the impact of antioxidants on oxidative stability of encapsulated shrimp.

1.3.7 To investigate the effects of micro-encapsulated shrimp oil fortification on the characteristics and sensory property of bread.

1.5 Flow chart of experiments



CHAPTER 2

Lipids from cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*): compositions and deterioration as affected by iced storage

2.1 Abstract

Lipids from cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) stored in ice for up to 6 days were extracted and characterized. The extraction yields of lipids from hepatopancreas (10.65–12.64%) were higher than those from cephalothorax (2.59–2.88%). However, no changes in the extraction yield were observed during the storage ($p > 0.05$). The carotenoid contents of lipids from cephalothorax and hepatopancreas slightly increased within the first 2 and 4 days of iced storage ($p < 0.05$), respectively, but decreased thereafter ($p < 0.05$). With increasing storage time, a progressive formation of hydroperoxide was found as evidenced by the increase in the absorbance band at 3600–3200 cm^{-1} in Fourier transform infrared (FTIR) spectra, and increased peroxide values (PVs) ($p < 0.05$). The increases in thiobarbituric acid reactive substances (TBARS), p-anisidine value (AnV) and free fatty acid (FFA) content of lipids were noticeable when iced storage time increased ($p < 0.05$). Those changes indicated that lipid oxidation and hydrolysis occurred in both samples. Phospholipids (PL) were the major components in lipids from cephalothorax (82.51% of total lipids). Nevertheless, lipids from hepatopancreas contained triglyceride (TG) and PL as the dominant components (45.35% and 38.03% of total lipids, respectively). A decrease in the TG content with a concomitant increase in free fatty acid was observed at the end of storage (day 6) ($p < 0.05$). Decreases in unsaturated fatty acids, especially eicosapentaenoic acid (EPA; C20:5(n-3)) and docosahexaenoic acid (DHA; C22:6(n-3)) were noticeable at day 6 of storage ($p < 0.05$). Thus, the extended storage time resulted in the enhanced deterioration of extracted lipids.

2.2 Introduction

Thailand is a world leading exporter of cultivated shrimp and its major importers are USA, followed by the EU, Japan, Canada and South Korea. The demand for shrimp is increasing yearly. Frozen shrimp is the top export item among frozen seafoods. In 2008, the production of 507,500 tons of Thai farmed marine shrimp was reported (FAO, 2010). During the industrial processing of shrimp, approximately 40–50% of its total weight are generated as by-products including cephalothorax, shell, etc. (Sachindra *et al*., 2005). Hepatopancreas, removed from shrimp cephalothorax, is another value byproduct generated from the manufacturing of whole shrimp, excluding hepatopancreas. Generally, shrimp byproducts are processed into animal feed and as protein feedstuff in aquaculture diets (Nwanna *et al*., 2004; Sudaryono *et al*., 1996). Apart from protein, chitin, minerals and carotenoids, lipids have received increasing interest as an important source of n-3 fatty acids, mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which have been known for their health benefit (Harper and Jacobson, 2001). In addition to fish oil, lipids from crustacean have gained increasing attraction, more likely due to their high content of carotenoids. Oil can be extracted from krill, a shrimp-like crustacean (Sampalis *et al*., 2003). The total lipid contents in Antarctic krill (*Euphausia superba*) ranged from 15% to 30% (dry weight basis) and increased with maturity. *Euphausia crystallorophias* had a significantly higher lipid content than E. superba (Ju and Harvey, 2004). Lipids extracted from salmon (*Salmo salar*) heads with the aid of commercial protease showed the yield of 19.6%, while the yield of lipids obtained from cooking method was lower (14.5%) (Gbogouri *et al*., 2006). Similar to fish oil, krill oil contains over 30% of EPA (C 20:5, n-3) and DHA (C 22:6, n-3). It also has high astaxanthin content (200–400 ppm) (Kolakowska *et al*., 1994). Astaxanthin exhibits 10-fold higher antioxidant activity, compared with other carotenoids, such as zeaxanthin, lutein, canthaxanthin and β-carotene and 100-fold greater than that of α-tocopherol (Miki, 1991). It is mainly used as a dyeing agent in the diets of aquacultured salmon and other species, but is also used in the cosmetic and pharmaceutical industries (Higuera-Ciapara *et al*., 2006). Recently,

carotenoprotein has been recovered from black tiger shrimp cepharothorax (Sowmya *et al*., 2011).

Shrimp byproduct is a highly perishable material (Gram and Dalgaard, 2002). Simultaneously, the loss in the nutritional value and the formation of toxic compound can occur. Deterioration of lipid as well as carotenoid more likely takes place during storage prior to extraction. Many factors including the degree of unsaturation of oil, the type and concentrations of antioxidants, pro-oxidants, moisture content, oxygen availability, temperature and degree of exposure to light have been known to affect the deterioration of lipids or oils (Bórquez *et al*., 1997; Chantachum *et al*., 2000; Coxon *et al*., 1987; Robards *et al*., 1988). However, no information regarding the changes in lipids and carotenoids in hepatopancreas and cephalothorax from Pacific white shrimp during handling and storage has been reported.

2.3 Objective

To investigate the changes of lipids and carotenoid in lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in iced.

2.4 Materials and methods

2.4.1 Chemicals

Palmitic acid, cupric acetate, ρ-anisidine, ammonium thiocyanate and pyridine 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, petroleum ether, hydrochloric acid, sulphuric acid and ammonium thiocyanate were purchased from Lab-Scan (Bangkok, Thailand). Astaxanthin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.4.2 Collection and preparation of cephalothorax and hepatopancreas from Pacific white shrimp

Cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) (Figure 4) were obtained from the Sea Wealth Frozen Food Co., Ltd., Songkhla province, Thailand. The shrimp with the size of 50-60 shrimp/kg were cultured in a farm in Songkhla province during September and October, 2011. Pooled cephalothorax (3-5 kg) and hepatopancreas (3-5 kg) were placed in 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 Songkla University, Hat Yai, Songkhla within approximately 2 h. All samples were divided into 200 g portions and placed in polyethylene bags. The bags were placed and distributed uniformly between the layers of ice. Insulated boxes containing samples and ice (1:2, w/w) were kept in a walk-in cold room (4 ºC) for 6 days. To maintain the sample/ice ratio, molten ice was removed and replaced every 2 days. During storage, the samples were randomly taken as the composite samples at day 0, 2, 4 and 6 for lipid extraction. Prior to lipid extraction, cephalothorax and hepatopancreas were ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec. Cephalothorax contained 75.35 ± 0.24% moisture, 4.17 ± 0.07% ash, 3.73 ± 0.34% fat and 14.54 ± 0.20% protein as determined by AOAC method (AOAC, 2000). Hepatopancreas consisted of 70.12 ± 0.74% moisture, 1.93 ± 0.02% ash, 11.79 ± 0.41% fat and 15.88 ± 0.77% protein.

Shrimp hepatopancreas

Shrimp cephalothorax

Figure 4 Hepatopancreas and cephalothorax of Pacific white shrimp

2.4.3 Lipid extraction

Lipid was extracted by the Bligh and Dyer method (Bligh and Dyer, 1959). Sample (25 g) was homogenized with 200 ml of a chloroform: methanol: distilled water mixture (50:100:50, v/v/v) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 ºC. The homogenate was added with 50 ml of chloroform and homogenized at 9500 rpm for another 1 min. Thereafter, 25 ml of distilled water were added and homogenized at the same speed for 30 sec. The homogenate was centrifuged at 3000g at 4 ºC for 15 min and transferred into a separating flask. The chloroform phase was drained off into the 125 ml Erlenmeyer flask containing 2-5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper (Whatman International Ltd., Maidstone, England). The solvent was evaporated at 25 ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. The yield was calculated and expressed as the percentage of lipids extracted (dry weight basis). Lipid samples were collected in a vial, flushed with nitrogen gas, sealed tightly and kept at -40 °C until analyses.

2.4.4 Analyses

2.4.4.1 Measurement of total carotenoid content

Total carotenoid content in the lipid samples was determined according to the method of Saito and Regier (1971) with a slight modification. Lipid (30 mg) was mixed with 10 ml of petroleum ether and the mixture was allowed to stand for 30 min. The absorbance of the extract, appropriately diluted, was measured at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration (C) of carotenoid in the sample was calculated using the equation given by Saito and Regier (1971) with a slight modification as follows:

$$\text{C (µg/glipid)} = \frac{\text{A468} \times \text{volume of extract} \times \text{dilution}}{0.2 \times \text{weight of sample used in gram}}$$

where 0.2 is the A468 of 1 µg /ml standard asthaxanthin

2.4.4.2 Measurement of lipid oxidation

2.4.4.2.1 Peroxide value (PV)

PV was determined using the ferric thiocyanate method (Chaijan *et al*., 2006). To 50 µl of lipid sample (10-fold dilution using 75% ethanol, v/v), 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 and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer. Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

2.4.4.2.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Lipid sample (0.5 g) 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 heated in a boiling water (95-100 ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600xg at 25 ºC for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was measured 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.

2.4.4.2.3 p-anisidine value (AnV)

AnV of sample was analysed according to the method of AOCS (AOCS, 1990).

2.4.4.3 Measurement of lipid hydrolysis by free fatty acid assessment

Free fatty acid (FFA) content, used as an index of hydrolysis, was determined according to the method of Lowry and Tinsley (1976). Lipid sample (0.1 g) was added with 5 ml of isooctane and swirled vigorously to dissolve the sample. The mixture was then added with 1 ml of 5% (w/v) cupric acetate-pyridine reagent, 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 for 90 sec using a Vortex-Genie2 mixer (Bohemia, NY, USA) and allowed to stand for 20 sec. The upper layer was subjected to absorbance measurement at 715 nm. A standard curve was prepared using palmitic acid in isooctane at concentrations ranging from 0 to 50 μmol/5 ml. FFA content was expressed as g FFA/100 g lipid.

2.4.4.4 Determination of lipid compositions

Lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored for 0 and 6 days in ice were determined for lipid compositions.

2.4.4.4.1 Lipid classes

Types of lipids were analysed using a thin layer chromatography/flame ionization 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 scanned twice before use in order to remove possible contaminants from the rods. The lipid samples dissolved in chloroform were spotted on the rod. Lipid classes were separated using a double development procedure with the following solvent systems: n-hexane: diethyl ether: formic acid

(50:20:0.3, 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 ºC) for 10 min before being analysed with the 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 (Senphan and Benjakul, 2012)

2.4.4.4.2 Fatty acid profile

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

2.4.4.4.3 Fourier transform infrared (FTIR) spectra

FTIR analysis of lipids was performed in a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, lipid sample (200 µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 4000–500 cm^{-1} (mid-IR region) with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC. Analysis of spectral data was carried out

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

2.4.5 Statistical analysis

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

2.5 Results and discussion

2.5.1 Changes in yield of lipids from cephalothorax and hepatopancreas

Yields and recovery of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp are shown in Table 3. Yield and recovery of lipid from hepatopancreas were much higher than that from cephalothorax ($p < 0.05$). During the iced storage, yields of lipids extracted from cephalothorax and hepatopancreas ranged from 10.52 to 11.68% and from 35.63 to 38.95% (dry weight basis), respectively. The results indicated that no marked changes in extraction yield and recovery were found throughout 6 days of storage ($p > 0.05$). The mixtures of cod (*Gadus morhua*) containing viscera showed the highest lipid recovery (up to 82.8% of total lipids), compared with those without viscera (Dauksas *et al*., 2005). Due to the higher content of lipid in hepatopancreas, the recovery of lipid was expected to be higher than that of cephalothorax. Additionally, hepatopancreas was the major source of protease (Oh *et al*., 2000), which might hydrolyze protein associated with lipid. As a result, the higher yield was found in lipid from hepatopancreas. Lipid content vary with species, seasons, feed, etc.

Table 3 Yield and recovery of lipids extracted from cephalothorax and hepatopancreas stored in ice for different times

Storage time (days)	Yield (% dry weight)		Recovery (% dry weight)	
	Cephalothorax	**Hepatopancreas**	**Cephalothorax**	**Hepatopancreas**
0	10.52 ± 0.71‡a*	35.63 ± 1.20a	84.19 ± 5.65a	90.26 ± 4.05a
2	10.82 ± 0.30a	38.40 ± 2.23a	86.60 ± 2.40a	97.30 ± 3.11a
4	11.68 ± 0.96a	37.37 ± 1.90a	93.52 ± 7.65a	94.67 ± 4.81a
6	11.62 ± 0.59a	38.95 ± 2.25a	93.04 ± 4.72a	98.68 ± 5.70a

‡ Values are given as means ± SD from triplicate determinations.

*Different lowercase letters in the same column indicate significant differences ($p < 0.05$)

2.5.2 Changes in carotenoid content

Carotenoid content of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for up to 6 days is presented in Figure 5. Carotenoids, especially astaxanthin, the main pigment found in crustacean and salmonids, provide the desirable reddish-orange color in these organisms (Higuera-Ciapara *et al*., 2006; Shahidi and Synowiecki, 1991). Carotenoid content of lipids from cephalothorax and hepatopancreas increased within the first 2 and 4 days of iced storage ($p < 0.05$), respectively. Total carotenoid contents of lipids extracted from cephalothorax and hepatopancreas stored for 2 and 4 days were 3.10 ± 0.02 and 1.89 ± 0.05 mg/g lipid, respectively. The carotenoid content in the wastes from Indian shrimp was found to vary from 35 to 153 μg/g, depending on their habitat or manufactured diets. The major pigment was astaxanthin and its esters (Sachindra *et al*., 2005). This result suggested that free carotenoids, especially astaxanthin, could be released from the protein matrix of both portions. During storage, hydrolysis caused by endogenous proteases, might facilitate the liberation of free carotenoid, from the proteinaceous matrix. As a result, the increase in carotenoid content was obtained. Furthermore, the exogenous proteases, especially from microorganisms, could be involved in hydrolysis of proteinaceous matrix, particularly when the storage time

increased. Thereafter, a decrease in carotenoid content was noticeable ($p < 0.05$). The decreased carotenoid content observed with extended storage time was presumed to be due to the enhanced oxidation of carotenoids. Carotenoids contain a high number of conjugated double bonds, which are susceptible to oxidation. Oxidation generally leads to discoloration of carotenoid (Choubert and Baccaunaud, 2006). During storage, cephalothorax and hepatopancreas underwent fading and turned to be more yellowish (data not shown). The color changes are in part caused by photooxidation of astaxanthin (Christophersen *et al*., 1991) and are enhanced by high partial pressure of oxygen (Nielsen *et al*., 1996). Carotenoid is sensitive to heat, oxygen, and light because of its unsaturated structure. As a consequence, carotenoid is gradually oxidized during storage (Ribeiro *et al*., 2005). Therefore, storage time directly affected stability of carotenoids in raw material, thereby determining the final content of carotenoid in the extracted lipids.

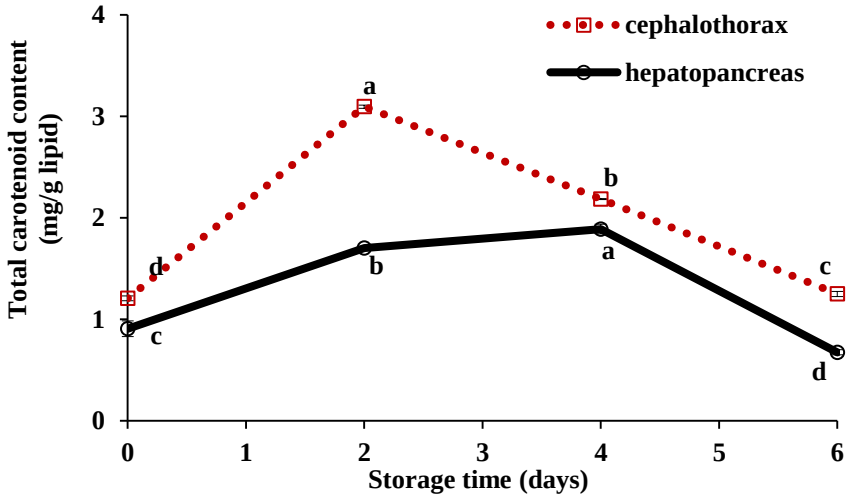


Figure 5 Carotenoid contents of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp during iced storage of 6 days. Different letters within the same sample indicate significant difference ($p < 0.05$). Bars represent standard deviations (n=3).

2.5.3 Lipid oxidation

2.5.3.1 Peroxide value (PV)

Changes in PV of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp during 6 days of iced storage are presented in Figure 6A. The continuous decrease in PV was observed in lipid extracted from cephalothorax throughout 6 days of iced storage ($p < 0.05$). For lipids extracted from hepatopancreas, PV increased within the first 2 days of iced storage ($p < 0.05$). Subsequently, a decrease in PV was noticeable up to day 6 ($p < 0.05$). The increase in PV of lipid from hepatopancreas was more likely due to the formation of hydroperoxide. The difference in PV of lipid extracted from two different raw materials was plausibly governed by several intrinsic factors, e.g. pro-oxidants, fatty acid composition, etc. The decrease in PV observed with extended storage time was due to the decomposition of hydroperoxide to the secondary oxidation products (Boselli *et al*., 2005). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalysed oxidation of polyunsaturated fatty acids (Nawar, 1996). The result indicated that lipid oxidation occurred in cephalothorax and hepatopancreas during iced storage, probably owing to the high content of unsaturated fatty acids. As a result, those lipid oxidation products were present in the extracted lipids and could contribute to quality of lipids.

2.5.3.2 TBARS

TBARS value of lipid from cephalothorax slightly decreased within the first 4 days of storage ($p < 0.05$) (Figure 6B). However, the increase was found at day 6 ($p < 0.05$). For hepatopancreas, the increase in TBARS was observed after 2 days of storage ($p < 0.05$). Nevertheless, no differences in TBARS were noticeable during 4 – 6 days of storage ($p > 0.05$). The initial values of TBARS for lipids extracted from cephalothorax and hepatopancreas were 10.78 and 26.55 mg MDA/kg sample, respectively, indicating that the lipid oxidation occurred during post-mortem handling, storage or extraction. The increase in TBARS value of lipids indicated the formation of the secondary lipid oxidation products (Chaijan *et al*., 2006). TBARS

have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). Marine lipids have been known to contain high content of PUFA (Tocher and Harvie, 1988). Those PUFA are prone to oxidation as indicated by the presence of TBARS in the lipids. The increases in TBARS were coincidental with the decrease in PV. This was probably due to the destruction of hydroperoxides into the secondary oxidation products, especially aldehydes, in the later stages of lipid oxidation. Additionally, the loss in natural antioxidants during extended storage might contribute to the increased lipid oxidation (Chaijan *et al*., 2006). Thus, lipid oxidation became more pronounced in cephalothorax and hepatopancreas when iced storage time increased. This resulted in the deterioration and unacceptability of extracted lipids.

2.5.3.3 ρ-anisidine value (AnV)

AnV of lipids from cephalothorax and hepatopancreas increased as the storage time increased ($p < 0.05$) (Figure 6C). In general, the similar trend was observed in comparison with TBARS values. It was noted that higher AnV was found in lipid extracted from cephalothorax, while the higher TBARS value was noticeable in lipid extracted from hepatopancreas ($p < 0.05$). It was suggested that lipid from cephalothorax contained a higher amount of non-volatile lipid oxidation product than that from hepatopancreas. AnV determines the amount of non-volatile aldehyde (principally 2-alkenals and 2,4-alkadienals) in lipid (Choe and Min, 2006). Aldehydes in lipid react with the ρ-anisidine reagent under acidic conditions. The reaction of ρ-anisidine with aldehydes yields yellowish products. Typically, AnV increases as aldehydes are produced. Those aldehydes can be further oxidized or participated in dimerization or condensation reactions (Gulla and Waghray, 2011). Increased AnV reconfirmed the generation of secondary oxidation products, mainly non-volatile compounds, which determined the quality of lipid. Navarro-García *et al*. (2010) reported that AnV of liver oil from two commercial rajiform species (*Rhinoptera bonasus* and *Aetobatus narinari*) increased as the storage time increased ($p < 0.05$). Thus, lipid oxidation products were formed in both raw materials, leading to the

presence of those products in the extracted lipids. However, types of lipid oxidation products varied, depending on raw materials used for extraction.

2.5.4 Lipid hydrolysis

Changes in free fatty acid content of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp during iced storage of 6 days are depicted in Figure 7. FFA content of lipids from hepatopancreas of Pacific white shrimp sharply increased after 2 days of storage ($p < 0.05$). Hydrolysis of glycerol–fatty acid esters is one important change that occurs in lipids, causing the release of free fatty acids. This is catalysed by lipases and phospholipases (Pacheco-Aguilar *et al*., 2000). Hepatopancreas is the major source of lipase, which is able to hydrolyze ester bond of triglyceride (Phillips *et al*., 1984). After 6 days of storage in ice, FFA content of lipid extacted from hepatopancreas was 92.95 g/100g, suggesting that lipids were almost complteley hydrolyzed. The result confirmed that hepatopancreas contained the active lipase or phospholipase, which were able to hydrolyze the lipids effectively with the extended iced stroage. For cephalothorax, no change in FFA content was observed within the first 4 days ($p > 0.05$) but a slight increase was noticeable thereafter ($p < 0.05$). At the end of storage period, lipid hydrolysis occurred to a great extent as evidenced by the highest FFA content obtained in lipids from both cephalothorax and hepatopancreas. Since both cephalothorax and hepatopancreas were stored in ice, lipases might be released and hydrolyzed triglyceride and phospholipids in both raw materials. In addition, extracellular lipase, produced by certain microorganisms, such as *Pseudomonas fragi*, was reported to contribute to the lipolytic breakdown of fish lipids (Nayak *et al*., 2003). Free fatty acids released were likely prone to oxidation. This led to the promoted lipid oxidation as indicated by increases in TBARS and AnV value, particularly at the end of storage.

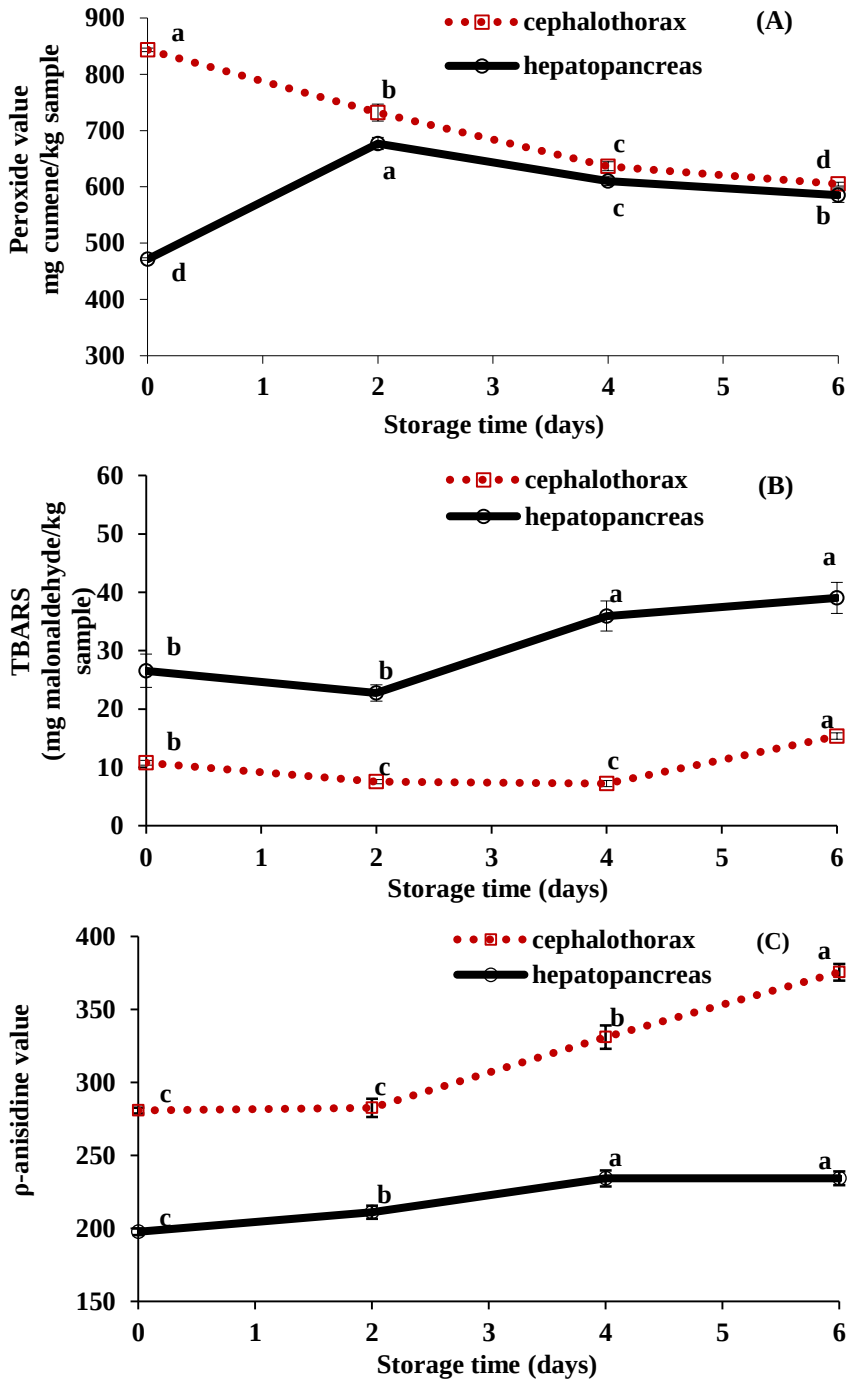


Figure 6 Peroxide values (A), TBARS values (B) and p-anisidine values (C) of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp during iced storage of 6 days. Different letters within the same sample indicate significant difference ($p < 0.05$). Bars represent standard deviations (n=3).

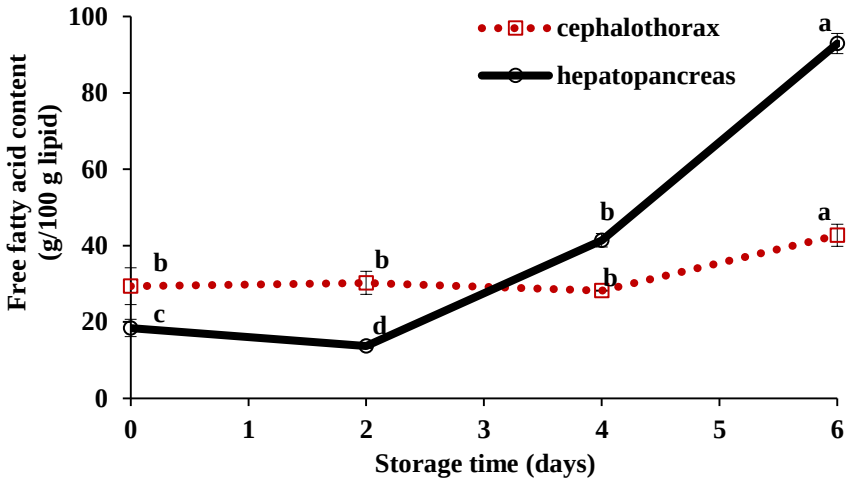


Figure 7 Free fatty acid contents of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp during iced storage of 6 days. Different letters within the same sample indicate significant difference ($p < 0.05$). Bars represent standard deviations (n=3).

2.5.5 Lipid compositions

2.5.5.1 Lipid classes

Compositions of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice at day 0 and day 6 are shown in Table 4. At day 0, lipid from cephalothorax had PL as the major constituent, accounting for 82.51% of total lipid, followed by TG and DG, representing 8.88 and 5.08% of total lipid, respectively. For lipid extracted from hepatopancreas, TG and PL were the major components, accounting for 45.35 and 38.03% of total lipid, respectively. FFA at a level of 8.71% was found, while small contents of DG and MG were noticeable. Differences in lipid compositions have been reported among individual species and maturation stage (Bledsoe *et al*., 2003; Sahena *et al*., 2009). Ju *et al*., (2004) reported that adult Antarctic krill (*E. superba*) contained TG as the dominant storage lipid, 45.5% of total lipid. The difference in lipid components between the lipids extracted from both raw materials more likely contributed to the difference in properties and stability. When lipids were extracted from raw materials stored in ice for 6 days, both TG and PL in lipids from hepatopancreas decreased with

concomitant increases in FFA, DG and MG contents ($p < 0.05$). The results suggested that TG and PL were hydrolyzed into free fatty acids, DG and/or MG during extended storage in ice. For lipid extracted from cephalothorax stored in ice for 6 days, no TG was detectable and PL content markedly decreased. Coincidentally, DG, MG and FFA contents increased ($p < 0.05$). The results suggested that TG was hydrolyzed to DG, MG and FFA during extended storage in ice. The increase in FFA in both lipid samples extracted from both raw materials stored in ice for 6 days was in agreement with the increase in FFA thoughtout the storage of 6 days (Figure 7). Lipids from cephalothorax and hepatopancreas were more likely susceptible to hydrolysis caused by lipase or phospholipase. Lipase and phospholipase in shrimp play an important role in hydrolysis of lipids (Lopez and Maragoni, 2000). Pasquevich *et al*., (2011) indicated that lipase is a potentially important determinant of the specific fatty acids released from the storage depots in hepatopancreas of crustaceans (*Macrobrachium borellii*). Our result was in agreement with Sikorski *et al*. (1990) who reported that about 20% of fish lipids were hydrolyzed during iced storage. Those free fatty acids released were prone to oxidation. As a result, lipid oxidation could proceed more rapidly, as shown by the pronounced increase in TBARS (Figure 6B) and AnV (Figure 6C). Therefore, the longer storage time negatively affected the extracted lipids, in which lipids underwent hydrolysis to a higher extent and were valuerable to deterioration, especially oxidation.

2.5.5.2 Fatty acid profiles

Fatty acid profiles of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for 0 and 6 days are shown in Table 5. At day 0, lipid from cephalothorax contained 29.51% SAT, 25.91% MUFA and 39.30% PUFA, whereas lipid from hepatopancreas consisted of 28.51% SAT, 29.95% MUFA and 37.42% PUFA. PUFAs in both samples were found as the major fatty acids. This result was in agreement with Lin *et al*., (2003) who found that PUFAs were the major fatty acids in white shrimp. Some differences in fatty acid compositions were noticeable between both lipids. Lipids from cephalothorax contained linoleic acid (C18:2(n-6)) as the most abundant fatty acid, followed by palmitic acid (C16:0), oleic acid (C18:1(n-9)). For hepatopancreas, linoleic acid was

Table 4 Composition of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for 0 and 6 days

Storage time (days)	Raw material	Composition (% of total lipid content)				
		TG	FFA	DG	MG	PL
0	Cephalothorax	8.88 ± 0.85 ‡B†	2.68 ± 0.12Bb*	5.08 ± 0.64Aa	0.84 ± 0.54Bb	82.51 ± 1.92Aa
	Hepatopancreas	45.35 ± 3.9Aa	8.71 ± 1.3Ab	4.12 ± 0.63Ab	3.79 ± 0.26Ab	38.03 ± 5.71Ba
6	Cephalothorax	ND	9.11±1.71Ba	2.69 ± 0.84Ba	1.94 ± 0.31Ba	86.26±2.73Aa
	Hepatopancreas	24.45 ± 2.31b	32.39 ± 1.88Aa	7.53 ± 0.51Aa	7.79 ± 0.32Aa	27.84 ± 3.90Ba

‡ Values are given as means ± SD from triplicate determinations.

† Different uppercase letters in the same column within the same day indicate significant differences ($p < 0.05$).

* Different lowercase letters in the same column within the same sample indicate significant differences ($p < 0.05$).

ND: non-detectable.

the dominant fatty acid, followed by oleic acid and palmitic acid. It was noted that lipids from hepatopancreas had the higher contents of palmitic acid, oleic acid and linoleic acid, but lower contents of stearic acid (C18:0), docosahexaenoic acid (C22:6(n-3), DHA) and eicosapentaenoic acid (C20:5(n-3), EPA) than those from ceophalothorax. Lipid from cephalothorax had higher contents of both DHA and EPA than those from hepatopancreas. Lipids from cephalothorax had EPA and DHA contents of 4.65 and 8.34 g/ 100 g oil, respectively, whereas EPA and DHA at levels of 2.15 and 6.20 g/100 g oil, respectively, were found in lipids from hepatopancreas. Chaijan *et al*., (2006) reported that DHA is usually more abundant than EPA (up to 2-3 times) in marine lipids. DHA in lipids extracted from cephalothorax and hepatopancreas were 1.79 and 2.88-fold higher than EPA. It has been reported that environmental and physiological factors affect the level of PUFA in marine lipids (Eghtesadi-Araghi and Bastami, 2011). Additionally, the extraction methods and solvent used also had the impact on fatty acid composition in krill oil (Lambertsen and Braekkan, 1971). Krill oil from Antarctic krill (*Euphausia superba*) was rich in both EPA and DHA (Maki *et al*., 2009). The amount of n-3 PUFAs of whole krill accounted for 19.0 ± 1.7% of fatty acid (Bustos *et al*., 2003). The high content of DHA was coincidental with the high content of phospholipid, which normally contain a high amount of PUFAs (Table 5). The n-3 fatty acids are anti-inflammatory, anti-thrombosis, anti-arrhythmia and reduce the lipid content of the blood, having vascular-dilatory properties (Sahena *et al*., 2009; Simopoulos, 2003). Thus the lipids present in cephalothorax and hepatopancreas of Pacific white shrimp are a rich source of n-3 fatty acids, offering benefits to human health.

When lipids were extracted from hepatopancreas and cepthalothorax stored in ice for 6 days, slight changes in fatty acid profiles were found. Nevertheless, DHA and EPA decreased by 1.94 and 4.56%, respectively, for lipid from cephalothorax and by 0.93 and 14.84%, respectively, for lipid from hepatopancreas. Thiansilakul *et al*., (2010) reported that the decreases in DHA and EPA contents suggested their susceptibility to oxidation during the extended storage. During iced storage, TG and PL underwent hydrolysis into free fatty acids (Figure 7), which were more likely prone to oxidation (Figure 6). As a result, n-3 fatty acids could be decreased, caused by those deteriorative processes.

Table 5 Fatty acid profiles of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for 0 and 6 days

Fatty acids (g/100 g oil)	Cephalothorax		Hepatopancreas	
	Day 0	Day 6	Day 0	Day 6
C10:0	ND	ND	0.02 ± 0.00a	ND
C12:0	ND	0.03 ± 0.02a	0.02 ± 0.01a	0.02 ± 0.01a
C14:0	0.61 ± 0.02‡b*	0.67 ± 0.01a	0.93 ± 0.18a	0.94 ± 0.08a
C14:1	0.12 ± 0.02a	0.13 ± 0.04a	0.18 ± 0.01a	0.18 ± 0.02a
C15:0	0.38 ± 0.01a	0.39 ± 0.10a	0.49 ± 0.03a	0.49 ± 0.02a
C15:1	0.63 ± 0.03a	0.63 ± 0.01a	0.13 ± 0.01a	0.13 ± 0.01a
C16:0	18.9 ± 0.04b	19.1 ± 0.06a	20.51 ± 0.02a	20.54 ± 0.04a
C16:1 *n*-7	1.44 ± 0.00a	1.45 ± 0.02a	1.99 ± 0.03a	2.01 ± 0.03a
C17:0	0.89 ± 0.02a	0.89 ± 0.01a	0.65 ± 0.01a	0.64 ± 0.02a
C17:1	0.3 ± 0.05a	0.29 ± 0.02a	0.33 ± 0.00b	0.34 ± 0.00a
C18:0	6.92 ± 0.03b	7.02 ± 0.04a	4.14 ± 0.04a	4.06 ± 0.01b
C18:1 *n*-9	18.66 ± 0.03b	18.83 ± 0.01a	21.76 ± 0.01a	21.74 ± 0.00b
C18:1 *n*-7	2.46 ± 0.02a	2.47 ± 0.01a	2.74 ± 0.03a	2.69 ± 0.01b
C18:2 *n*-6	19.69 ± 0.02a	19.70 ± 0.02a	23.28 ± 0.07b	23.40 ± 0.02a
C18:3 *n*-3	1.29 ± 0.00b	1.32 ± 0.01a	1.71 ± 0.01a	1.73 ± 0.04a
C18:3 *n*-6	0.02 ± 0.01a	0.03 ± 0.00a	0.03 ± 0.02a	0.03 ± 0.04a
C18:4 *n*-3	0.09 ± 0.00a	0.09 ± 0.03a	0.11 ± 0.01a	0.11 ± 0.02a
C20:0	0.32 ± 0.1a	0.32 ± 0.02a	0.33 ± 0.02a	0.32 ± 0.03a
C20:1 *n*-7	0.18 ± 0.03a	0.18 ± 0.00a	0.24 ± 0.02a	0.24 ± 0.01a
C20:1 *n*-9	1.00 ± 0.02a	1.00 ± 0.02a	1.21 ± 0.04a	1.20 ± 0.02a
C20:1 *n*-11	0.37 ± 0.01a	0.36 ± 0.00a	0.55 ± 0.02a	0.55 ± 0.03a
C20:2 *n*-6	1.78 ± 0.00a	1.75 ± 0.01b	1.73 ± 0.00b	1.76 ± 0.02a
C20:3 *n*-6	0.10 ± 0.00b	0.11± 0.00a	0.13 ± 0.01a	0.13 ± 0.02a
C20:3 *n*-3	0.22 ± 0.03a	0.22 ± 0.01a	0.24 ± 0.02a	0.25 ± 0.02a
C20:4 *n*-6	2.34 ± 0.02a	2.30 ± 0.01b	0.96 ± 0.01a	0.91 ± 0.02b
C20:4 *n*-3	0.14 ± 0.01a	0.13 ± 0.05a	0.17 ± 0.03a	0.18 ± 0.02a
C20:5 *n*-3 (EPA)	4.65 ± 0.01a	4.56 ± 0.02b	2.15 ± 0.00a	2.13 ± 0.01b
C21:0	0.12 ± 0.02a	0.12 ± 0.04a	0.15 ± 0.00a	0.15 ± 0.02a
C22:0	0.31 ± 0.01a	0.29 ± 0.00b	0.29 ± 0.04a	0.28 ± 0.02a
C22:1 *n*-9	0.10 ± 0.02a	0.10 ± 0.03a	0.13 ± 0.01a	0.13 ± 0.03a
C22:1 *n*-11, *n*-13	0.35 ± 0.01a	0.33 ± 0.02a	0.52 ± 0.02a	0.51 ± 0.01a
C22:2	ND	ND	0.02 ± 0.00a	0.02 ± 0.00a

Table 5 Fatty acid profiles of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for 0 and 6 days (Cont.)

Fatty acids (g/100 g oil)	Cephalothorax		Hepatopancreas	
	Day 0	Day 6	Day 0	Day 6
C22:4 *n*-6	0.17 ± 0.01a	0.17 ± 0.00a	0.21 ± 0.01a	0.20 ± 0.01a
C22:5 *n*-6	0.47 ± 0.01a	0.45 ± 0.00b	0.48 ± 0.05a	0.48 ± 0.02a
C22:6 *n*-3 (DHA)	8.34 ± 0.02a	7.96 ± 0.02b	6.20 ± 0.05a	5.28 ± 0.04b
C23:0	0.19 ± 0.00a	0.18 ± 0.01a	0.22 ± 0.01a	0.22 ± 0.03a
C24:0	0.87 ± 0.02a	0.82 ± 0.02b	0.76 ± 0.02a	0.76 ± 0.02a
C24:1	0.30 ± 0.01a	0.28 ± 0.01a	0.17 ± 0.03a	0.17 ± 0.01a
Saturated fatty acid (SFA)	29.51 ± 0.04b	29.83 ± 0.02a	28.51 ± 0.04a	28.42 ± 0.02b
Monounsaturated fatty acid (MUFA)	25.91 ± 0.05b	26.05 ± 0.02a	29.95 ± 0.02a	29.89 ± 0.02b
Polyunsaturated fatty acid (PUFA)	39.30 ± 0.02a	38.97 ± 0.01b	37.42 ± 0.04a	36.61 ± 0.02b

‡ Values are given as means ± SD from triplicate determinations.

* Different lowercase letters in the same row within the same sample indicate significant differences ($P < 0.05$).

ND: non-detectable.

2.5.5.3 FTIR spectra

FTIR spectra of lipids from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice at day 0 and day 6 are illustrated in Figure 8. Dominant peaks were found at a wavenumber range of 3050–2800 cm^{-1}, representing CH stretching vibrations, which overlap with –OH group in carboxylic acids (3100–2400 cm^{-1}). A higher amplitude of peak with wavenumber of 3600–3100 cm^{-1} representing –OH, –NH, ≡CH and =C–H stretching was observed in lipid from cephalothorax, compared with hepatopancreas counterpart, regardless of storage time. This was possibly due to the higher amounts of DG and PL in lipids from cephalothorax (Table 4).Hydroperoxide moieties exhibit the characteristic absorbance peak between 3600 and 3400 cm^{-1} due to their –OO–H stretching vibrations (Van de Voort *et al*., 1994). A larger peak representing hydroperoxide was found in lipids from cephalothorax than that from hepatopancreas. The result was confirmed by the

higher PV in the former (Figure 6A). It was noted that the higher amplitude was found in lipids extracted from cephalothorax and hepatopancreas stored in ice for 6 days, compared with those extracted from fresh samples (day 0). 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 oxidation process. When a ratio of $A_{2854}/A_{3600–3100}$ was determined, a slight change was found in lipids from cephalothorax stored for 6 days in ice. This could be explained by the lower oxidation rate. Nevertheless, a higher ratio of $A_{2854}/ A_{3600–3100}$ was observed in lipids from hepatopancreas stored in ice for 6 days, indicating the advanced oxidation process. This was concomitant with a rapid increase in TBARS of lipids extracted from hepatopancreas at day 6 of storage (Figure 6B). After 6 days of iced storage, the higher amplitude of peak at the wavenumber of 2853 cm^{-1} was observed in both lipid samples. The increased amplitude in this region indicated the higher amounts of aldehyde formed in both samples at the end of storage. Peak observed at 3400 cm^{-1} was due to their –OO–H stretching vibrations (Van de Voort *et al*., 1994). A larger peak representing hydroperoxide was found in lipids from cephalothorax, compared with that of lipids from hepatopancreas. The result confirmed the slightly higher PV in lipids extracted from cephalothorax (Figure 6A).

Generally, the ester carbonyl functional group of TG was observed at the wavenumber of 1741–1746 cm^{-1} (Setiowaty *et al*., 2000). Peaks at wavenumbers of 1743 and 1745 cm^{-1} were considered to be a TG peak for lipids from cephalothorax and hepatopancreas, respectively. After 6 days of storage, a shift towards lower wavenumbers was found in both samples. These changes were more likely associated with an increase in peak amplitude with a wavenumber of 1711 cm^{-1}, which represents the C=O carboxylic group of free fatty acids (Guillen and Cabo, 1997). The increase in absorbance at 1711 cm^{-1} suggested the accumulation of FFA for both samples as shown in Figure 7. This observation was coincidental with the increase in FFA determined by TLC/FID, as shown in Table 4. The peaks found at 1166 and 1237 cm^{-1} were associated with the stretching vibration of the C–O ester groups and the bending vibration of CH_2 groups, and the peaks observed at 1117 and 1095 cm^{-1} represented the stretching vibration of the C–O ester groups. Guillen and Cabo (2004)

reported that the changes in the ratios of A_{2854}/A_{1237}, A_{2854}/A_{1163}, A_{2854}/A_{1117} or A_{2854}/A_{1099} could be used to indicate the oxidation degree of the samples. After 6 days of storage, lipids extracted from cephalothorax exhibited the greater changes of these ratios than those from hepatopancreas. The lower ratio of A_{2854}/A_{1237}, representing C–O–C in ethers and esters, was found in both samples after 6 days of storage. The result suggested that advanced lipid oxidation and hydrolysis occurred in cephalothorax and hepatopancreas with increasing storage time.

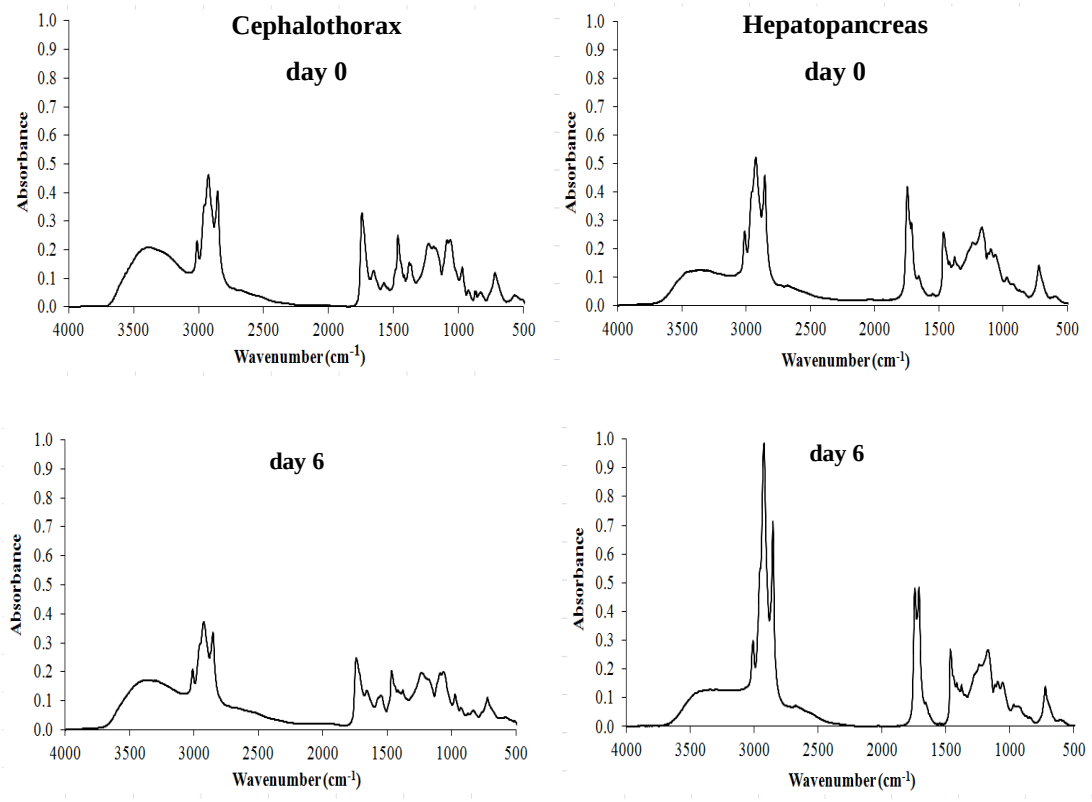


Figure 8 FTIR spectra of lipids extracted from cephalothorax and hepatopancreas of Pacific white shrimp stored in ice for 0 and 6 days.

2.6 Conclusion

Cephalothorax and hepatopancreas of Pacific white shrimp were susceptible to lipid oxidation and hydrolysis during iced storage. Hydroperoxides were generated and underwent decomposition to yield the secondary oxidation products, especially aldehydes. An increase in hydrolysis, indicated by the formation

of FFA and the concomitant decrease in TG, was observed throughout the storage period. Those lipid changes showed a detrimental effect on the quality of lipids extracted from cephalothorax and hepatopancreas. Therefore, the suppression of lipid oxidation and hydrolysis could be a means to maintain the quality of lipids extracted from raw material stored in ice.

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CHAPTER 3

Extraction and stability of carotenoid containing lipid from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*)

3.1 Abstract

Impacts of extraction conditions on lipid and carotenoid yields from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) were investigated. Among single solvents (acetone, isopropanol and hexane) and their mixtures, a mixture of hexane and isopropanol (50: 50, v/v) rendered lipid with the highest carotenoid yield (336.40 mg/kg hepatopancreas) with the extraction yield of 18.22% (w/w hepatopancreas) ($p < 0.05$). The use of hepatopancreas to solvent ratio of 1: 4.5 (w/v) and three repetitions showed the highest carotenoid yield (363.94 mg/kg hepatopancreas) with the extraction yield of 18.08% (w/w hepatopancreas). Astaxanthin, astaxanthin diester and canthaxanthin were the major carotenoids found in the lipid. When astaxanthin (2 mg/g lipid) was added into lipid, the oxidation was lowered, in comparison with the control during the storage at 30 °C for 10 days as indicated by lower ρ-anisidine value. Fourier transform infrared (FTIR) spectra study also revealed that triglyceride in lipids underwent oxidation to a lower degree when astaxanthin was incorporated.

3.2 Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4’-dione), a liposoluble carotenoid from xanthophylls family, is the most abundant pigment in aquatic animals such as salmon, trout, shrimp and lobster (Higuera-Ciapara *et al*., 2006; Shahidi and Brown 1998). In general, animals are incapable of synthesising astaxanthin and acquire from the diet (Hussein *et al*., 2006). Astaxanthin used in aquaculture is mostly supplied by synthetic route. Due to the high cost of synthetic pigments, natural pigments have gained increasing attention for applications (Higuera-Ciapara *et al*., 2006). Astaxanthin has been reported as the main carotenoid found in shrimp

byproduct e.g. cephalothorax, shell, etc. (Sachindra *et al*., 2005). Recently, hepatopancreas, another byproduct generated from the manufacturing of whole shrimp excluding hepatopancreas, has been used as the excellent source of lipid with high polyunsaturated fatty acids (PUFA) (Takeungwongtrakul *et al*., 2012). Generally, shrimp byproducts are converted to protein feedstuff for aquaculture (Nwanna *et al*., 2004). Apart from protein, chitin, minerals and lipids, carotenoids have been paid increasesing interest as the important source of antioxidants (De Holanda and Netto 2006). Astaxanthin exhibits 10-fold higher antioxidant activity, compared with other carotenoids such as zeaxanthin, lutein, canthaxanthin and β-carotene and is 100-fold greater than that of α-tocopherol (Miki, 1991). It is mainly used as a dyeing agent in the diets of aquacultured salmon and other species. However, it is also used in the cosmetic and pharmaceutical industries (Higuera-Ciapara *et al*., 2006). There was a correlation between natural antioxidant content and the resistance to oxidation of oil from tecolote ray (Navarro-Garcıa *et al*., 2004).

To extract lipid and carotenoid, several solvents have been widely used. Organic solvents have been permitted for use in food industries like acetone, ethyl acetate, hexane, isopropanol, methanol, methyl ethyl ketone and ethanol. On the other hand, solvents such as dichloromethane, dimethyl sulfoxide and chloroform are not allowed, because of their toxicity (FDA, 2010). Carotenoids are generally soluble in non-polar solvents (Sachindra *et al*., 2006). The polar solvents such as acetone (Nègre-Sadargues *et al*., 2000) and a mixture of polar and non-polar solvents (Sachindra *et al*., 2006) were used to improve extraction yield of carotenoid from shrimp head and carapace. Sánchez-Camargo *et al*. (2011) reported that the mixture of hexane and isopropanol (40: 60, v/v) gave the highest carotenoid extraction yield (53 mg/kg waste) from the redspotted shrimp waste, compared to acetone (34 mg/kg waste). Nevertheless, no information regarding the extraction of carotenoid containing lipids from hepatopancreas of Pacific white shrimp and the role of astaxanthin in stabilization of lipids has been reported.

3.3 Objectives

To study the extraction conditions for carotenoid containing lipids from hepatopancreas of Pacific white shrimp.

To determine the role of astaxanthin in prevention of oxidaton in lipid extracted from hepatopancreas during extend storage.

3.4 Materials and methods

3.4.1 Chemicals

ρ-anisidine and ammonium thiocyanate were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, 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). Acetone, hexane, isopropanol, petroleum ether and hydrochloric acid were purchased from Lab-Scan (Bangkok, Thailand). Astaxanthin was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

3.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during November, 2011 and January, 2012. Pooled hepatopancreas (3-5 kg) was placed in polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18 °C until use, but the storage time was not longer than 1 month. Prior to extraction of carotenoid containing lipids, hepatopancreas was ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

3.4.3 Extraction of carotenoid containing lipids from hepatopancreas

3.4.3.1 Effect of different organic solvents

Carotenoid containing lipids in the hepatopancreas were extracted using various organic solvents and solvent mixtures following the method of Sachindra *et al*. (2006). Different organic solvents used included 1) hexane, 2) isopropanol, 3) acetone, 4) hexane: acetone (25: 75, v/v), 5) hexane: acetone (50: 50, v/v), 6) hexane: acetone (75: 25, v/v), 7) hexane: isopropanol (25: 75, v/v), 8) hexane: isopropanol (50: 50, v/v), 9) hexane: isopropanol (75: 25, v/v), 10) acetone: isopropanol (25: 75, v/v), 11) acetone: isopropanol (50: 50, v/v), and 12) acetone: isopropanol (75: 25, v/v).

To extract carotenoid containing lipids, the prepared hepatopancreas (20 g) was homogenized with 50 ml of cold organic solvent or solvent mixtures at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 °C. The extracts were filtered using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, England). The residue was extracted with corresponding solvent for another two times. For the extracts using isopropanol, acetone and their mixtures solvents, the filtrates were mixed with an equal quantity of petroleum ether. The petroleum ether fractions were repeatedly washed with an equal quantity of 0.1% NaCl in order to separate the phases and remove traces of polar solvents. All extracts were added with 2-5 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvents were evaporated at 40 °C using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing.

3.4.3.2 Effect of hepatopancreas/solvent ratio and repetition

The solvent rendering the highest extraction yield and astaxanthin content was used. Hepatopancreas to solvent ratios of 1: 2.5, 1: 4.5 and 1: 6.5 (w/v) and extraction repetition of 2, 3 and 4 times were used. The extraction was performed

in the same manner as previously described. The obtained extracts were calculated for yield and were subjected to analyses.

3.4.4 Analyses

3.4.4.1 Measurement of total carotenoid content

Total carotenoid content in the samples was determined according to the method of Saito and Regier (1971) with a slight modification. Sample (30 mg) was mixed with 10 ml of petroleum ether and the mixture was allowed to stand for 30 min. The absorbance of the extract, appropriately diluted, was measured at 468 nm using a UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). The concentration (C) of carotenoid in the sample was calculated using the equation given by Saito and Regier (1971) with a slight modification as follows:

$$\mathrm{C}\ (\mu g/\mathrm{glipid}) = \frac{\mathrm{A468}\ \times \text{volume of extract}\ \times \text{dilution}}{0.2\ \times \text{weight of sample used in gram}}$$

where 0.2 is the A468 of 1 µg/ml standard astaxanthin

3.4.4.2 Thin-layer chromatography

The sample extracted by the organic solvent or solvent mixture with the highest astaxanthin content was subjected to thin-layer chromatography (TLC) using activated 20 × 20 cm silica gel plates (Silica gel G. Merck, type 60, Darmstadt, Germany) following the modified procedure described by Sánchez-Camargo *et al*., (2011). The sample was applied to the plates and the separation was carried out using a mobile phase (acetone: hexane, 25: 75, v/v).

3.4.4.3 Role of astaxanthin in stabilization of lipid during extended storage

To elucidate the impact of astaxanthin on prevention of lipid oxidation, astaxanthin (2 mg/g lipid) was added into lipid and mixed well. Lipid containing indigenous carotenoids including astaxanthin (without astaxanthin addition) was used as the control. Samples with and without the addition of astaxanthin were kept in

amber bottle and capped tightly. The samples were stored at room temperature (28 – 30 °C) and were taken randomly for analyses at day 0, 2, 4, 6, 8 and 10.

3.4.4.3.1 Peroxide value (PV)

PV was determined using the ferric thiocyanate method (Chaijan *et al*., 2006). Carotenoid-containing lipid sample was diluted 10-flod using 75% ethanol (v/v). The solution (50 µl) was mixed with 2.35 ml of 75% ethanol (v/v), 50 µl of 30% ammoniumthiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 35% HCl (w/v) were added and mixed thoroughly After 3 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer. Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 mM. PV was expressed as mg cumene hydroperoxide/kg lipid.

3.4.4.3.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). The sample (0.5 g) was mixed with 2.5 ml of a solution containing 3.75% thiobarbituric acid (w/v), 15% trichloroacetic acid and 0.25 M HCl. The mixture was heated in a boiling water (95-100 °C) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600xg at 25 °C for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was measured at 532 nm using a spectrophotometer. A standard curve was prepared using 1,1,3,3-tetramethoxypropane, a precusor for malonaldehyde, at the concentrations ranging from 0 to 6 mM. TBARS were calculated and expressed as mg malonaldehyde/kg lipid.

3.4.4.3.3 ρ-Anisidine value(AnV)

AnV of sample was analysed according to the method of AOCS (AOCS, 1990). The sample (100 mg) was dissolved in 25 ml of isooctane. The solution (2.5 ml) was 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. The ρ -anisidine value was calculated using the following formula:

$$\rho-\text{anisidine value} = 25 \times \frac{(1.2 \times A_2) - A_1}{W}$$

where A_1 and A_2 are A_{350} before and after adding ρ-anisidine, respectively; W= weight of sample (g)

3.4.4.3.4 Fourier transform infrared (FTIR) spectra

FTIR analysis of samples stored for 0 and 10 days at 30 °C was performed in a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) equipped with a Bruker Model Vector 33 FTIR spectrometer (Bruker Co., Ettlingen, Germany). Prior to analysis, the crystal cell was cleaned with acetone, wiped dry with soft tissue and the background scan was run. For spectra analysis, sample (200 µl) was applied directly onto the crystal cell and the cell was clamped into the mount of the FTIR spectrometer. The spectra in the range of 4000 – 500 cm^{-1} (mid-IR region) with the automatic signal gain were collected in 16 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker Co., Ettlingen, Germany).

3.4.5 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie 1960). Statistical analysis was performed using the Statistical Package for Social Science (SPSS for windows, SPSS Inc., Chicago, IL, USA).

3.5 Results and discussion

3.5.1 Effect of different organic solvents on extraction of carotenoid containing lipid

Carotenoid containing lipid extracted from hepatopancreas of Pacific white shrimp using different solvents or mixtures showed varying yields (Table 6). All samples were reddish orange in color. Carotenoids, especially astaxanthin, provide the desirable reddish-orange color in crustaceans (Higuera-Ciapara *et al*., 2006; Shahidi and Synowiecki 1991). Among all solvents and mixtures used, hexane rendered the non-significantly lower yield of lipid, compared with isopropanol and acetone ($p > 0.05$). However, the mixture between hexane and isopropanol (50: 50, v/v) and the mixture between hexane and acetone (50: 50 and 25: 75, v/v) showed the higher yield than hexane alone ($p < 0.05$). It was noted that all solvents used except hexane rendered the similar yield ($p > 0.05$). Hexane is non-polar solvent, which might not be appropriate to extract lipid in hepatopancreas, in which phopholipid constituted as the major components (See chapter 2). Thus, the mixture between polar and non-polar solvents could extract phospholipid, which comprised both polar phosphate group and hydrocarbon non-polar domain. When comparing carotenoid content in the extracted lipids, all samples showed similar content, except for lipid extracted using hexane, which had the lowest content ($p < 0.05$). Carotenoid is soluble in lipids or oils, and constituted as the coloring pigment in obtained lipids. Among all extracted lipids, the highest yield of carotenoid was obtained when hexane: isopropanol (50: 50, v/v) was used as the solvent ($p < 0.05$), whereas hexane showed the lowest efficacy in extracting carotenoid ($p < 0.05$). The result was in agreement with Sachindra *et al*., (2006) who reported the highest carotenoid yield (43.9 mg/kg waste, wet weight basis) from head and carapace of *Penaeus indicus* when the carotenoid containing lipids were extracted with 50: 50 (v/v) mixture of hexane and isopropanol. The variation in carotenoids in crustaceans was probably caused by the differences in environmental conditions, species and other variables (Mezzomo *et al*., 2011). However, the mixture of acetone, hexane and isopropanol promoted carotenoid extraction from head and carapace of pink shrimp (Mezzomo *et al*., 2011). The use of

a mixture of polar and non-polar solvents for extraction of carotenoid from shrimp byproduct generally resulted in the highest yield (Khanafari *et al*., 2007). Since carotenoid could be soluble in lipid, it was co-extracted with lipid from hepatopancreas. However, it was noted that hexane: isopropanol (50: 50, v/v) mixture more likely had the appropriate polarity for carotenoid extraction, as evidenced by the highest carotenoid yield (336.40 mg/kg hepatopancreas). The use of hexane and isopropanol instead of acetone is beneficial in the large-scale extraction of carotenoid containing lipids from shrimp byproduct, as the cost of isopropanol and hexane are lower than that of acetone (Sachindra *et al*., 2006). The present study indicated that hexane and isopropanol mixture (50: 50, v/v) was the appropriate extraction solvent to extract lipid and to recover carotenoid from Pacific white shrimp hepatopancreas.

3.5.2 Effect of hepatopancreas/solvent and repetition on extraction of carotenoid containing lipid

Extraction yields of carotenoid containing lipid using the mixture of hexane and isopropanol (50: 50, v/v) at different hepatopancreas/solvent ratios and various repetitions are shown in Table 7. No differences in extraction yields were found for all extraction conditions used ($p > 0.05$). Lipids extracted with higher repetitions had the increases in carotenoid content, when hepatopancreas/solvent ratios of 1: 2.5 and 1: 4.5 (w/v) were used. Nevertheless, at a ratio of 1: 6.5 (w/v), repetitions had no effect on carotenoid content ($p > 0.05$). Similar result was observed for carotenoid yield. With lower hepatopancreas/solvent ratio and lower repetition, the use of hepatopancreas/solvent ratio of 1: 4.5 (w/v) and 3 repetitions was selected as the most appropriate condition to extract carotenoid containing lipid. Britton (1985) reported that tissues contain a large amount of water. The first extraction with polar solvents might remove little pigment, but the tissues were dried. Thus, carotenoid yield increased with the subsequent extractions. In the present study, a mixture of polar and non-polar solvents was used. Polar solvent removed the water in tissues, which aided in the extractability of pigments by non-polar solvents in subsequent extractions (Sachindra *et al*., 2006).

Table 6 Extraction yield, carotenoid content and carotenoid yield of lipids from hepatopancreas of pacific white shrimp as affected by solvents and solvent mixtures[*]

Solvents/Mixtures (v/v)	Extraction yield (g/100 g wet weight basis)	Carotenoid content (mg/g lipids)	Carotenoid yield (mg/kg hepatopancreas)
Hexane	7.46 ± 4.53[‡]b[†]	1.60 ± 0.10b	161.40 ± 3.74f
Iso-propanol	13.43 ± 4.28ab	1.71 ± 0.12ab	264.81 ± 29.36d
Acetone	10.33 ± 3.90ab	1.80 ± 0.08ab	226.20 ± 3.73e
Hexane :Acetone (75:25)	14.26 ± 4.17ab	1.70 ± 0.23ab	259.00 ± 21.05d
Hexane :Acetone (50:50)	17.45 ± 1.84a	1.76 ± 0.24ab	292.65 ± 20.01bc
Hexane :Acetone (25:75)	16.14 ± 3.09a	1.79 ± 0.15ab	301.20 ± 6.93b
Hexane :Iso-propanol (75:25)	14.74 ± 4.06ab	1.68 ± 0.35ab	220.87 ± 12.54e
Hexane :Iso-propanol (50:50)	18.22 ± 0.50a	1.83 ± 0.03a	336.40 ± 5.42a
Hexane :Iso-propanol (25:75)	13.15 ± 1.93ab	1.82 ± 0.04a	261.71 ± 5.05d
Acetone :Iso-propanol (75:25)	12.95 ± 3.99ab	1.87 ± 0.15a	274.35 ± 7.60cd
Acetone :Iso-propanol (50:50)	10.14 ± 3.33ab	1.84 ± 0.05a	227.47 ± 8.03e
Acetone :Iso-propanol (25:75)	10.42 ± 3.15ab	1.84 ± 0.08a	224.16 ± 5.69e

[‡] Values are given as means ± SD (n=3).

[†] Different letters within the same column denote significant differences ($p < 0.05$).

[*] Hepatopancreas/solvent ratio of 1: 2.5 (w/v), Repetition = 3

Table 7 Extraction yield, carotenoid content and carotenoid yield of lipids from hepatopancreas of pacific white shrimp as affected by hepatopancreas/solvent ratios and repetitions[*]

Hepatopancreas to solvent ratio (w/v)	Repetitions	Extraction yield (g/100 g wet weight basis)	Carotenoid content (mg/g lipids)	Carotenoid yield (mg/kg hepatopancreas
1: 2.5	2	17.51 ± 2.21[‡]a[†]	1.70 ± 0.11c	279.10 ± 14.36d
	3	18.11 ± 1.43a	1.82 ± 0.03bc	334.17 ± 5.21c
	4	19.83 ± 0.49a	1.92 ± 0.08ab	343.50 ± 13.18bc
1: 4.5	2	17.70 ± 0.93a	1.85 ± 0.14bc	331.16 ± 14.00c
	3	18.08 ± 0.77a	2.02 ± 0.22ab	363.94 ± 8.41ab
	4	19.28 ± 1.04a	2.09 ± 0.03a	378.95 ± 37.85a
1: 6.5	2	18.12 ± 0.52a	1.96 ± 0.02ab	347.90 ± 3.82bc
	3	19.59 ± 0.99a	2.08 ± 0.19a	368.62 ± 14.44ab
	4	19.39 ± 0.66a	2.03 ± 0.13ab	369.70 ± 12.50ab

[‡] Values are given as means ± SD (n=3).

[†] Different letters within the same column denote significant differences ($p < 0.05$).

[*] Hexane/isopropanol mixture (50:50, v/v) was used as extraction solvent.

3.4.3 Identification of carotenoids in lipids

When being separated by TLC, carotenoids in lipid extracted from hepatopancreas of Pacific white shrimp showed six distinct bands at retention factor (R_f) of 0.33, 0.40, 0.44, 0.60, 0.66 and 0.75, respectively (Figure 9). Very small band was detected at R_f of 0.96. The band with R_f of 0.33 corresponded to free astaxanthin, while bands with R_f of 0.96 was identified as β-carotene. The orange bands having R_f of 0.40, 0.50 and at 0.75 corresponded to canthaxanthin, astaxanthin monoester and astaxanthin diester, respectively (Lorenz-Todd, 1998). The results indicated that free astaxanthin, canthaxanthin, and astaxanthin diester were the major pigments in lipids extracted from hepatopancreas of Pacific white shrimp. TLC is a technique widely used to separate and purify carotenoids due to its simplicity, flexibility and low cost (Sánchez-Camargo *et al*., 2011). The lipids containing those pigments were therefore used for pigmentations of shrimp or other crustaceans (Hussein *et al*., 2006). Shrimp pigmentation is caused by keto-carotenoids (astaxanthin and canthaxanthin). These keto-carotenoids contain a conjugated double bond system, responsible for their color. Antioxidant activity of astaxanthin has been shown to be related to the number of conjugated double bonds (Conn *et al*., 1991).

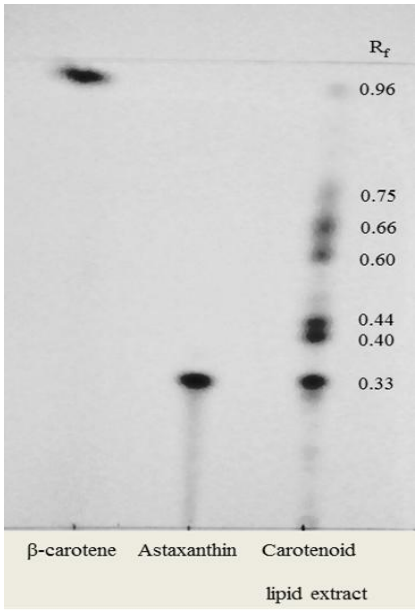

Figure 9 Thin layer chromatography of carotenoids in lipids from hepatopancreas of Pacific white shrimp.

3.5.4 Stability and antioxidative role of carotenoids in lipids from pacific white shrimp

3.5.4.1 Peroxide value (PV)

Changes in PV of carotenoid containing lipids extracted from hepatopancreas with and without added astaxanthin (2 mg/g lipid) during 10 days of storage are presented in Figure 10A. During the first 2 days of storage, the sample added without astaxanthin had the higher PV ($p < 0.05$). Subsequently, a decrease in PV was noticeable in sample without the addition of astaxanthin up to day 10 ($p < 0.05$). However, the sample added with astaxanthin had the gradual increase in PV throughout 10 days of storage ($p < 0.05$). The great change in PV of carotenoid containing lipids was also observed during 10 days of storage, probably due to its high PUFA content. PUFA content of lipids in the present study was higher than that found in lipid from the belly flaps of Nile perch (22.75 g/100 g) (Ogwok *et al*., 2008) and in lipid from sardine muscle (35.7 g/100 g) (Chaijan *et al*., 2006). In addition, hepatopancreas contains high amount of lipase (Pasquevich *et al*., 2011). Thus, lipase might accelerate the hydrolysis of lipids, thereby yielding higher free fatty acids, which were more prone to oxidation. The increase in PV of sample was more likely due to the formation of hydroperoxide. For the sample without astaxanthin addition, the decrease in PV with extended storage was due to the decomposition of hydroperoxide to the secondary oxidation products (Boselli *et al*., 2005). Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalysed oxidation of polyunsaturated fatty acids (Nawar, 1996). The sample without added astaxanthin underwent oxidation faster than that with added astaxanthin. The result indicated that astaxanthin played a crucial role as antioxidant in lipids extracted from Pacific white shrimp hepatopancreas. Astaxanthin was reported to be 10-fold stronger than that of other carotenoids, namely zeaxanthin, lutein, canthaxanthin and carotene (Naguib, 2000) and 100-fold greater than that of α-tocopherol (Miki, 1991). Carotenoids undergo decomposition when they behave as antioxidants. They prevent the oxidation of lipids, by making themselves available for reactions with radicals instead of lipids

(Sowmya and Sachindra, 2012). Therefore, the indigenous astaxanthin in lipid extracted from hepatopancreas could improve the stability of lipids during storage.

3.5.4.2 TBARS

TBARS value of carotenoid containing lipids extracted from hepatopancreas with and without added astaxanthin increased as the storage time increased up to 10 days ($p < 0.05$) (Figure 10B). The initial TBARS value of sample with and without added astaxanthin was 12.30 mg MDA/kg sample, indicating that lipid oxidation occurred during post-mortem handling, storage or extraction. The increase in TBARS value of both samples indicated the formation of the secondary lipid oxidation products (Chaijan *et al*., 2006). TBARS have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). The lipids from hepatopancreas of Pacific white shrimp were a rich source of polyunsaturated fatty acids (PUFAs) (Senphan and Benjakul, 2012; Takeungwongtrakul *et al*., 2012). Lipids from hepatopancreas consisted of 28.51 g/100 g saturated fatty acid, 29.95g/100 g monounsaturated fatty acid and 37.42 g/100 g PUFAs. Those PUFAs were prone to oxidation as indicated by the presence of TBARS in both samples. The increases in TBARS were coincidental with the decrease in PV, especially for the sample without addition of astaxanthin. Additionally, the loss in natural antioxidants, particularly astaxanthin, during extended storage might contribute to the increased lipid oxidation (Chaijan *et al*., 2006). When comparing TBARS values between both samples, that with added astaxanthin contained the higher TBARS value than that without added astaxanthin during the 10 days of storage ($p < 0.05$). This was probably due to the greater loss in volatile oxidation products with low molecular weight of the latter during the extended storage (Maqsood *et al*., 2012).

3.5.4.3 ρ-Anisidine value (AnV)

AnV of carotenoid containing lipids extracted from hepatopancreas with and without added astaxanthin increased when the storage time increased ($p < 0.05$) (Figure 10C). The addition of astaxanthin lowered the formation of the

oxidation products and the generation of secondary oxidation products, mainly non-volatile compounds. AnV is a measure of the production of aldehydes during oxidation of lipid and can be used as an indicator of the oxidative history of the lipid (O'Sullivan *et al*., 2005). The reaction of ρ-anisidine with aldehydes under acidic condition yields yellowish products. Typically, AnV increases as aldehydes are produced. AnV of the sample without added astaxanthin was significantly higher than that of sample with added astaxanthin throughout the storage ($p < 0.05$). Astaxanthin has been reported to be efficient in protection of unsaturated lipids against oxidation. It plays a role as a scavenger of radicals involved in lipid autoxidation (Andersen *et al*., 1990). The results reconfirmed the antioxidative activity of astaxanthin found in carotenoid containing lipids. Also, it indicated that the addition of astaxanthin effectively prevented the oxidation of lipid from Pacific white shrimp hepatopancreas.

3.5.5 FTIR spectra

FTIR spectra of carotenoid containing lipid extracted from hepatopancreas of Pacific white shrimp stored at 30 °C for 0 and 10 days are shown in Figure 11. Dominant peaks were found at a wavenumber range of 3050–2800 cm^{-1}, representing CH stretching vibrations, which overlap with –OH group in carboxylic acids (3100–2400 cm^{-1}). Amplitude of peak with wavenumber of 3600–3100 cm^{-1} frepresenting –OH, –NH, ≡CH and =C–H stretching was also observed in all samples. The result reconfirmed that lipid from hepatopancreas contained triglycerides and phospholipids as the dominant components (Takeungwongtrakul *et al*., 2012). Hydroperoxide moieties exhibit the characteristic absorption peak between 3600 and 3400 cm^{-1} due to their –OO–H stretching vibrations (Van de Voort *et al*., 1994). At day 10 of storage, the hydroperoxide band at wavenumber of 3396 cm^{-1} in the sample with added astaxanthin showed a higher amplitude, compared to that of sample without astaxanthin addition. This was coincidental with the results of PV (Figure 10A). The hydroperoxide formed in the sample without added astaxanthin might undergo the intensive decomposition, yielding the secondary lipid oxidation products, especially low molecular weight (MW) volatile compounds.

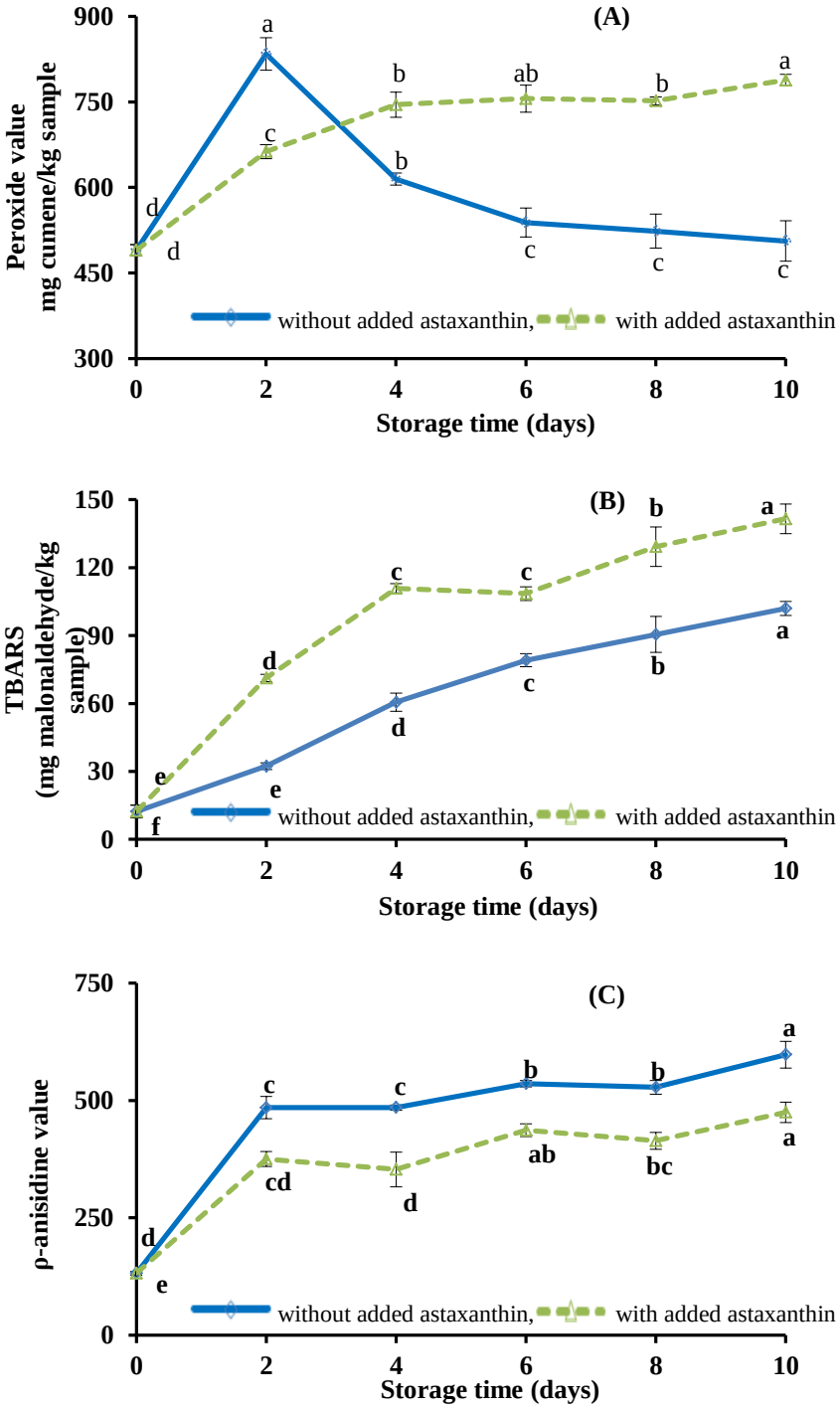

Figure 10 Peroxide values (A), TBARS values (B) and *ρ*-anisidine values (C) of lipids extracted from hepatopancreas of Pacific white shrimp during iced storage of 10 days. Different letters within the same sample indicate significant difference ($p < 0.05$). Bars represent standard deviations (n=3).

On the other hand, the sample with added astaxanthin still had the accumulated hydroperoxide at day 10. This result indicated that astaxanthin lowered the decomposition of hydroperoxide. 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 point out the changes in oxidation process. When a ratio of $A_{2854}/A_{3600–3100}$ was determined, a slight change was found in the sample without added astaxanthin after 10 days of storage. This could be explained by the lower oxidation rate. Nevertheless, a higher ratio of $A_{2854}/A_{3600–3100}$ was observed in the sample added with astaxanthin after 10 days of storage, indicating the presence of oxidation products. This was concomitant with a rapid increase in TBARS of the sample with added astaxanthin at day 10 of storage (Figure 10B). Volatile oxidation products with low MW could be lost during extended storage. The increased amplitude in this region indicated the higher amounts of aldehyde formed in both samples at the end of storage. Generally, the ester carbonyl functional group of triglycerides was observed at the wavenumber of 1741–1746 cm^{-1} (Setiowaty *et al*., 2000). Peak at wavenumbers of 1741 cm^{-1} was considered to be a triglyceride peak of lipids from hepatopancreas. After 10 days of storage, a shift towards lower wavenumbers was found in both samples. The peak amplitude of the sample with added astaxanthin was higher than that without added astaxanthin. The result indicated that the higher amounts of triglycerides were retained in the sample with added astaxanthin, compared with that found in the sample without added astaxanthin. The result suggested that triglycerides were prevented from oxidation process by astaxanthin, both indigenous and added astaxanthins.

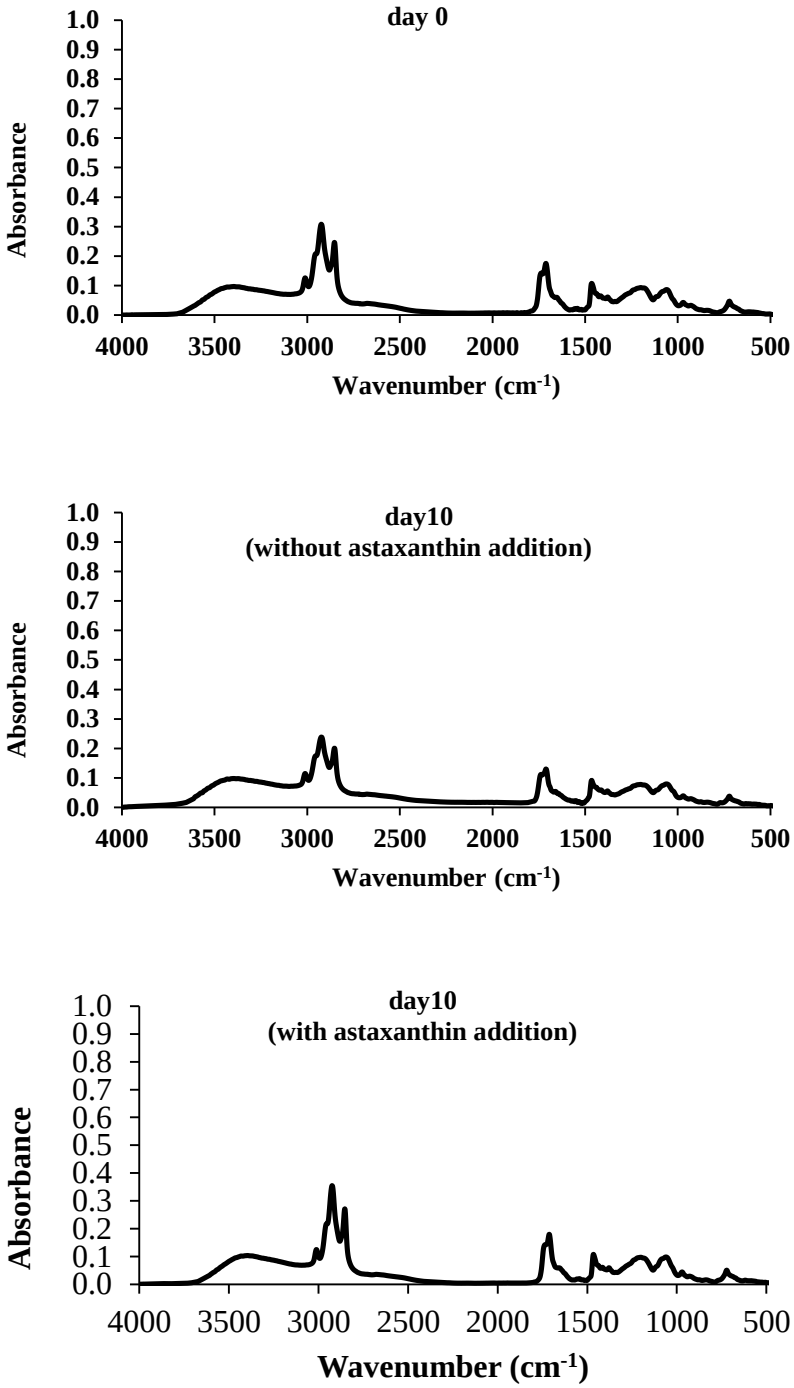

Figure 11 FTIR spectra of carotenoid containing lipids extracted from hepatopancreas of Pacific white shrimp at day 0 and those without and with astaxanthin addition at day 10 of storage at 30 °C.

3.6 Conclusions

Extraction of carotenoid containing lipids from hepatopancreas of Pacific white shrimp could be successfully done using the mixture of hexane and isopropanol (50: 50, v/v) with hepatopancreas/solvent ratio of 1: 4.5 (w/v) for three times. The process was the cost-effective and rendered the highest carotenoid yield. Carotenoids in lipids contained astaxanthin, astaxanthin diester and canthaxanthin as the major constituents. The addition of astaxanthin in the lipids prevented the oxidation during storage at 30 °C for 10 days. Carotenoid containing lipids can therefore be used as the valuable product for health care.

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CHAPTER 4

Oxidative stability of lipid from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) as affected by essential oils incorporation

4.1 Abstract

Oxidative stability of lipid from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) added with different essential oils at 200 ppm during 10 days of storage was studied. With increasing storage time, the progressive formation of hydroperoxide was found in all samples as evidenced by the increases in peroxide values (PV) ($p < 0.05$). Amongst all essential oils tested, lemon essential oil exhibited the highest preventive effect toward oxidation, followed by plai and basil essential oils, respectively. The increases in thiobarbituric acid reactive substances (TBARS) and ρ-anisidine value (AnV) of lipids were noticeable when storage time increased ($p < 0.05$). The increases in TBARS and AnV were retarded in the presence of essential oils. Nevertheless, the effectiveness for prevention of lipid oxidation of all three essential oils was slightly lower than α-tocopherol when used at the same level. The formation of volatile compounds and rancid odor of samples were lowered when being incorporated with essential oils. Lipid containing lemon essential oil had the similar rancid odour to that added with α-tocopherol using the sensory evaluation. Thus, essential oils could be used as the potential natural antioxidant to retard lipid oxidation and rancidity of lipids from hepatopancreas during extended storage.

4.2 Introduction

Lipids from crustacean have gained increasing attraction, more likely due to their high content of carotenoids. Hepatopancreas, a byproduct generated from the manufacturing of hepatopancreas-free whole shrimp is the excellent source of lipids with high polyunsaturated fatty acids (PUFA) and astaxanthin (Takeungwongtrakul *et al*., 2012). Nevertheless, lipids from hepatopancreas are known to be susceptible to oxidation. Lipid oxidation has been reported to be responsible for the formation of off-flavor, and toxic compounds as well as the cause

of many diseases (Shaker, 2006). Lipid oxidation is the major cause of rancidity, which is associated with limited shelf-life of food and other products. To retard or prevent oxidative deterioration, antioxidants, especially synthetic ones, have been employed widely. However, they may cause the negative side effect for consumers (Lim *et al*., 2011). Hence, there is a tendency towards the use of natural antioxidants, particularly from plant origin to replace with those synthetic antioxidants (Zhang *et al*., 2010).

Essential oils are oily aromatic liquids extracted from aromatic vegetable plants and widely used in foods (Burt, 2004). Essential oils are composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes (Isman *et al*., 2007). Additionally, essential oils have been reported to possess antioxidative activity and reduce the oxidation (Fasseas *et al*., 2008; Singh *et al*., 2012). Thailand has a variety of aromatic plant, herb or spices, which can serve as the source of essential oils. Basil, plai and lemon essential oils are of interest since they are abundant in Thailand. Those essential oils can be used as natural antioxidant in lipids with high PUFA content. Nevertheless, no information regarding the impact of essential oils from tropical plants on prevention of lipid oxidation of lipids from hepatopancreas shrimp has been reported.

4.3 Objective

To elucidate the effect of different essential oils on lipid oxidation of lipids from hepatopancreas of Pacific white shrimp during storage.

4.4 Materials and methods

4.4.1 Chemicals

ρ-anisidine and ammonium thiocyanate were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, 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). Hexane, isopropanol, and hydrochloric acid were purchased

from Lab-Scan (Bangkok, Thailand). Essential oils (100% purity) from lemon, plai and basil were obtained from Botanicessence (Bangkok, Thailand).

4.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during March, 2013 and May, 2013. Pooled hepatopancreas (3-5 kg) was placed in polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18°C until use and the storage time was not longer than 1 month. Prior to extraction of lipids, hepatopancreas was ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

4.4.3 Extraction of lipid from hepatopancreas

Lipid was extracted from hepatopancreas following the method in chapter 3. The prepared hepatopancreas (20 g) was homogenized with 90 ml of a cold solvent mixture of isopropanol and hexane (50:50, v/v) at a speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 ºC. The extracts were filtered using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, England). The residue was extracted with the cold solvent mixture for another two times. The hexane fractions were pooled and repeatedly washed with an equal quantity of 1% NaCl in order to separate the phases and remove traces of polar solvents. Hexane fraction was then added with 2-5 g of anhydrous sodium sulfate, shaken thoroughly, and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvents were evaporated at 40 ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing.

4.4.4 Oxidative stability of lipids as affected by different antioxidants

The lipid was added with lemon, plai and basil essential oils at a level of 200 ppm. Sample added with α-tocopherol (200 ppm) was also prepared. Sample without addition of essential oils or α-tocopherol was used as the control. All samples with and without the additions of antioxidants were transferred into amber bottles and capped tightly. The samples were stored at 30±1 ºC in an incubator (Memmert, D-91126, Schwabach, Germany) and drawn randomly for analyses at day 0, 2, 4, 6, 8 and 10.

4.4.5 Analyses

4.4.5.1 Peroxide value (PV)

PV was determined using the ferric thiocyanate method (Chaijan *et al*., 2006). To 50 µl of lipid sample (10-fold dilution using 75% ethanol, v/v), 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 and mixed thoroughly. After 3 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide/kg sample.

4.4.5.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Lipid sample (0.5 g) 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 heated in a boiling water (95-100 ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600xg at 25 ºC for 20 min using a centrifuge (Hettich mikro 20, Tuttlingen, Germany). The absorbance of the supernatant was measured 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 sample.

4.4.5.3 ρ-anisidine value (AnV)

AnV of sample was analysed according to the method of AOCS, (1990). The sample (100 mg) was dissolved in 25 ml of isooctane. The solution (2.5 ml) was 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. The ρ-anisidine value was calculated using the following formula:

$$\rho\text{– anisidine value} = 25 \times \frac{(1.2 \times A_2) - A_1}{W}$$

where A_1 and A_2 are A_{350} before and after adding ρ-anisidine, respectively; W= weight of sample (g)

4.4.5.4 Measurement of volatile compounds

The volatile compounds in different essential oils (lemon, plai and basil essential oils) were determined using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Iglesias and Medina (2008) with a slight modification.

Lipid (without essential oils) at day 0 was also determined for volatile compounds. Volatile compounds in lipid samples added without and with different essential oils and α-tocopherol, were measured after 10 days of storage using SPME GC-MS.

4.4.5.4.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 1 g of sample was mixed with 4 ml of deionised water and stirred continuously to disperse the sample. The mixture was heated at 60 ºC in 20 ml headspace vial with equilibrium time of 1 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC 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 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

4.4.5.4.2 GC–MS analysis

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). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

4.4.5.4.3 Analyses of volatile compounds

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). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid

oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

4.4.5.5 Evaluation of essential oil and rancid odor

Lipid samples containing essential oils were prepared for panelist training using Generic descriptive analysis (Meilgaard *et al*., 1999). The sensory panel (10-15 panelists) familiar with essential oil odor and rancid odor was screened for perception of odor and the ability to determine differences between samples (International Standard ISO 8586-1, 1993; ASTM Special Technical Publication 758, 1981; Meilgaard, 1999).

During training, the panelists attended 3 h training sessions during 1 week period. Reference odor samples (14 ml) were placed in a sealable plastic cup. Panelists were asked to open the sealable cup and sniff the headspace above the samples for determining essential oil and rancid odor intensity. Those odors were discussed among panelists in an open session until agreement was reached on the final intensity definition. Panelists were asked to rate for intensities of individual odor on a 150 mm unstructured scale, anchored with the terms ‘not very’ at the low end and ‘very’ at the high end. The reference of each essential oil was prepared by addition of essential oil in the lipids to obtain the concentrations of 0, 200 and 400 ppm, representing the score of 0, 8 and 15, respectively. For rancid odor, the reference was prepared by incubating lipids at 40ºC for 0, 5, 10 and 15 days, representing the score of 1, 5, 10 and 14, respectively.

For testing the panelists were asked to evaluate for the essential oil odor and rancid odor of lipid samples. The samples (14 ml) were coded with random three-digit codes and served randomly to each panelist. The panelists were asked to sniff tissue paper before testing each sample. Intensity was scored on a 150 mm unstructured scale (Yarnpakdee *et al.*, 2012).

4.4.6 Statistical analysis

Experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was

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

4.5 Results and discussion

4.5.1 Oxidative stability of lipids added with different essential oils

Lipids added with lemon, basil and plai essential oils at a level of 200 ppm were oxidized at different degrees during 10 day-storage. Generally, those added with essential oils exhibited lower oxidation than control sample.

4.5.1.1 Peroxide value (PV)

Changes in PV of lipids from hepatopancreas of Pacific white shrimp incorporated with basil, lemon and plai essential oils in comparison with that added with α-tocopherol (200 ppm) during 10 days of storage are presented in Figure 12A. A continuous increase in PV was observed in all samples throughout 10 days of storage ($p < 0.05$). PV is a measure of the concentration of hydroperoxides formed at the initial stages of lipid oxidation. The increase in PV of all samples indicated that the samples were in propagation stage of lipid oxidation with a lower rate of decomposition of hydroperoxides formed. Lipid hydroperoxides are formed by various pathways including the reaction of singlet oxygen with unsaturated fatty acids or the lipoxygenase-catalysed oxidation of polyunsaturated fatty acids (Nawar, 1996).

When comparing PV of the samples, the control sample (without antioxidant) had the higher PV than others throughout 10 days of storage, followed by those incorporated with basil, plai and lemon essential oils, and α-tocopherol, respectively ($p < 0.05$). For the first 2 days of storage, PV in the sample added with α-tocopherol was not different from that found in the sample incorporated with lemon essential oil ($p > 0.05$). It was noted that there was no difference in PV between both samples at day 10 ($p > 0.05$). The results indicated that samples added with antioxidant exhibited a slower rate of hydroperoxide formation. However, the efficiency in prevention of lipid oxidation varied with essential oils used. Different

essential oils more likely contained varying antioxidative compounds with different radical scavenging activity, in which propagation stage was terminated.

4.5.1.2 TBARS

TBARS values of lipids incorporated with and without antioxidants for 10 days of storage are depicted in Figure 12B. TBARS values of all samples were seen to increase during the storage period ($p < 0.05$). The increase in TBARS value of lipids indicated the formation of the secondary lipid oxidation products (Chaijan *et al*., 2006). TBARS value is an index of lipid oxidation status and it is related with the malonaldehyde content of the sample (Papastergiadis *et al*., 2012). Hydroperoxides, the initial reaction products of PUFA, react with oxygen to form malonaldehyde, which may contribute to off-flavor of oxidised lipids (Zhang *et al*., 2010). Shrimp lipids are known to contain high content of PUFA (Takeungwongtrakul *et al*., 2012). Those PUFAs are prone to oxidation as indicated by the presence of TBARS in lipids, particularly with extended storage time. For all samples, the increasing rate of TBARS was high within the first 7 days of storage. Thereafter, the increase in TBARS was retarded. This might be caused by the loss in low molecular weight volatile secondary oxidation products in the samples.

Amongst all samples, the control sample showed the highest TBARS in comparison with other samples and that containing α-tocopherol had the lowest TBARS value throughout 10 day-storage ($p < 0.05$). Lemon, plai and basil essential oils were found to present preventive effects on TBARS formation in the decreasing order. Thus, it can be concluded that essential oils, particularly lemon essential oil, showed effectiveness in the prevention of the oxidation of lipid from hepatopancreas of Pacific white shrimp.

4.5.1.3 ρ-anisidine value (AnV)

AnV of lipids from hepatopancreas of Pacific white shrimp added with different essential oils increased when the storage time increased ($p < 0.05$) (Figure 12C). However, the rate of the increase varied with essential oils added. AnV is one of the oldest methods for evaluating the level of secondary lipid oxidation products. It

is based on the reactivity of the aldehyde carbonyl bond on the ρ-anisidine amine group, leading to the formation of a Schiff base with absorbance at 350 nm (Laguerre *et al*., 2007). Changes in AnV were similar to those of TBARS, mainly indicating the formation of volatile secondary products, as shown in Figure 12B. The addition of antioxidants lowered the formation of non-volatile secondary oxidation products, as evidenced by the decrease in AnV. AnV of the control sample was significantly higher than those of samples added with essential oils and α-tocopherol ($p < 0.05$). Lemon essential oil also showed the highest effectiveness in retarding the increase in AnV, compared with the other two essential oils. The results reconfirmed the antioxidative activity of all essential oils in lipids from Pacific white shrimp hepatopancreas.

4.5.2 Volatile compounds of different essential oils and their effect on the odor of lipids

Volatile compounds identified in different essential oils are shown in Table 8. There were 27 volatile compounds detected in the lemon essential oil, while plai essential oil and basil essential oil consisted of 23 and 18 compounds, respectively. The main constituents found in lemon essential oil were limonene, followed by citral, β-citral, α-pinene, α-citral, linalool, limonene oxide and carveol, respectively. The composition of lemon essential oil was in agreement with Di Vaio, Graziani *et al.* (2010) who reported that limonene was the major component of lemon essential oil. Plai essential oil contained sabinene as the major component, followed by 4-terpineol, β-sesquiphellandrene, γ-terpinene, α-terpinene, cymol, and α-terpinenyl acetate, respectively. Sukatta *et al.* (2009) also reported that sabinene was the major compound in plai essential oil. For basil essential oil, methyleugenol constituted the dominant component, followed by trans-caryophyllene, the dominant component, followed by trans-caryophyllene, α-humulene, borneol, germacrene D, eugenol and α-terpineol, respectively. Hussain *et al*. (2008) found that linalool was the main constituent of *O. basilicum* essential oil, followed by epi-α-cadinol, α-bergamotene, γ-cadinene, germacrene D and camphor, respectively. According to Freire *et al*. (2006), eugenol and 1,8-cineole were the main compounds in *Ocimum*

gratissimum leaves obtained in different seasons (Autumn, Winter, Spring and Summer). The differences in the constituents of basil essential oils across countries may be due to different environmental and genetic factors, different chemotypes and the nutritional status of the plants (Hussain *et al.,* 2008). Different volatile compounds determined in different essential oils were more likely associated with varying smells. When essential oils were mixed with lipids at a concentration of 200 ppm, the smell of the essential oils could be detected at day 0 (Table 9). However, the intensity of essential oil smell was seen to decrease after 10 days of storage. This was plausibly due to the loss in volatile components in essential oil during the storage.

4.5.3 Volatile compounds of lipids added with essential oils after the storage

Volatile compounds in lipids with and without lemon, plai and basil essential oils and α-tocopherol addition after 10 days of storage are displayed in Table 10. After the storage of 10 days, the control sample contained several new volatile compounds including 2-hexyl-1-octanol, 2-(5-methyl-5-vinyltetrahydro-2-furanyl) propanal and 3-octen-1-ol. The higher amount of lipid oxidation products such as 3-hexanol, 2-butyl-1-octanol, hexadecanal and 2-tert-butyl-4-isopropyl-5-methylphenol were found in the control sample after 10 days in comparison with those detected in the initial sample reported in chapter 2. However, 2-undecanol and 2,7-octadien-1-ol were obtained only in the initial sample. Lipids from hepatopancreas contained unsaturated fatty acids, in which linoleic acid (C18:2(n-6)) was the dominant fatty acid, followed by oleic acid (C18:1(n-9)) (See chapter 2). Those fatty acids are prone to oxidation. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acids (Varlet *et al*., 2006). After 10 days of storage, it was noted that 2-undecanol and 2,7-octadien-1-ol was not detected, plausibly due to the volatilization. The result indicated that higher lipid oxidation and greater decomposition of hydroperoxide occurred during preparation and storage. The formation of volatiles was reduced as the essential oils or α-tocopherol were incorporated in the lipids. Volatile compounds in the sample added with α-tocopherol were lowest in abundance, compared with those found in those added with essential

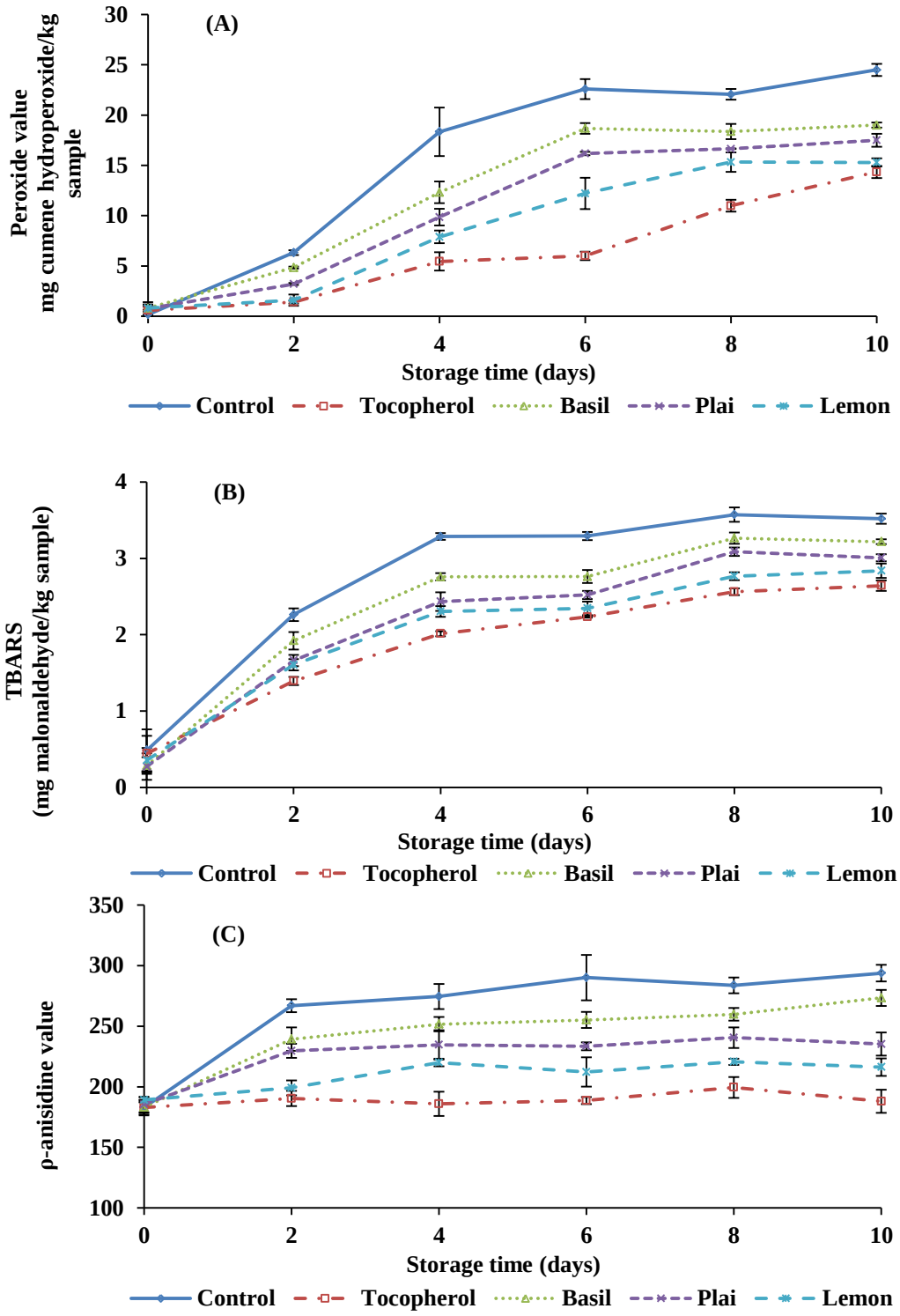

Figure 12 Peroxide values (A), TBARS values (B) and *p*-anisidine values (C) of lipids from hepatopancreas of Pacific white shrimp during 10 days of storage at 30 ºC. Different letters within the same sample indicate significant difference ($p < 0.05$). Bars represent standard deviations (n=3).

Table 8 The volatile compounds of lemon, plai and basil essential oils.

Compounds	**Peak area (Abandance) × 10^7**		
	Lemon essential oil	**Plai essential oil**	**Basil essential oil**
2,2-dimethyl-1,3-dioxane-4,6-dione	657	ND	ND
Vinyl methyl ether	ND	54	ND
α-pinene	3,551	739	ND
Sabinene	ND	5,928	ND
α-terpineol	ND	ND	630
α-terpinene	ND	2,363	ND
Limonene	6,564	1,458	ND
ρ-cymene	605	ND	200
ɣ-terpinene	192	4,136	ND
Cymol	ND	2,342	ND
α-terpinolene	249	1,444	ND
Trans sabinene hydrate	ND	560	ND
Limonene oxide	2,060	ND	ND
Phenylmethanal	ND	ND	84
β-limonene-1,2-epoxide	788	ND	ND
Cis-sabinene hydrate	ND	201	ND
Cis-ρ-2-menthen-1-ol	ND	572	ND
Linalool	2,238	ND	ND
Trans-caryophyllene	ND	ND	3,133
2,6,6-trimethyl-1-cyclohexene-1-acetaldehyde	247	ND	ND
4-terpineol	ND	5,208	ND
Myrtenal	715	ND	ND
Cis- ρ-menth-2,8-dienol	827	ND	ND
Trans-pinocarveol	819	ND	ND
ɣ-elemene	ND	222	ND
Piperitol isomer	ND	140	ND
Aromadendrene	ND	ND	87
Borneol	ND	ND	1,030
α-terpinenyl acetate	ND	1,250	ND
β-citral	4,351	ND	ND
α-humulene	ND	ND	1,250
Germacrene	ND	ND	982
Zingiberene	ND	524	ND
α-citral	2,801	ND	ND
δ-cadinene	ND	ND	221

Table 8 The volatile compounds of lemon, plai and basil essential oils (Cont.).

Compounds	**Peak area (Abandance) × 10^7**		
	Lemon essential oil	**Plai essential oil**	**Basil essential oil**
Citral	5,026	ND	ND
β-sesquiphellandrene	ND	4,539	ND
1-cyclohexene-1-carboxaldehyde, 4-(1-methylethenyl)-	144	ND	ND
ρ-cymen-7-ol	ND	46	ND
Carveol	1,680	ND	ND
Cis-carveol	972	ND	ND
Trans- ρ-mentha-1(7),8-dien-2-ol	112	ND	ND
Dodeca-1,6-dien-12-ol, 6,10-dimethyl-	152	ND	ND
10-limonenyl acetate	125	ND	ND
Perilla alcohol	214	ND	ND
Methyleugenol	ND	ND	6,830
(-)-caryophyllene oxide	ND	ND	9
Elemol	ND	ND	142
4-isopropyl-1-methyl-1,2,3-cyclohexanetriol	156	ND	ND
Eugenol	ND	ND	674
Cis-methyl isoeugenol	ND	ND	194
4a-methyl-trans-2-decalinone	185	ND	ND
α-eudesmol	ND	ND	61
Junipercamphor	ND	ND	75
4-isopropenyl-1-methyl-1,2-cyclohexanediol	271	ND	ND
2-allyl-1,4-dimethoxy-6-methylbenzene	ND	271	ND
Triquinacene, 1,4-bis(methoxy)-	ND	109	ND
Nerolic acid	109	ND	ND
Geranic acid	265	ND	ND
7-nitro-2,3-dimethylindole (2,3-dimethyl-7-nitroindole)	ND	164	ND
Vanillin	ND	ND	22
Acremine E	ND	41	ND
3,4-dimethoxycinnamaldehyde	ND	50	26

ND: non-detectable

Table 9. Essential oil odor of carotenoid containing lipid in the absence and presence of essential oil before and after storage for 10 days at 30 ºC.

Samples	**Day**	
	0	**10**
Lemon essential oil	4.35 ± 0.90‡A†	2.68 ± 0.71B
Plai essential oil	4.26 ± 0.77A	2.87 ± 0.48B
Basil essential oil	3.99 ± 0.69A	2.40 ± 0.81B

‡ Values are given as means ± SD (n=3).

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

oils. It was found that the types of compounds detected in lipids were different when different essential oils were incorporated. This was plausibly due to the difference in antioxidative activity of different essential oils as evidenced by varying formation of lipid oxidation products. During storage, essential oils could act as antioxidant, thereby lowing rancidity. Different essential oils contained different antioxidants, which were able to scavenge radicals at varying degrees (Tongnuanchan *et al*., 2013). Differences in physicochemical nature of the antioxidative components presented in the essential oils may contribute to varying antioxidative activity (Ahmadi *et al*., 2007). The antioxidant potential of phenolic compounds is dependent on the number and arrangement of the hydroxyl groups as well as the presence of electron-donating substituents in the ring structure (Kaisoon *et al*., 2011). They can act as antioxidants by donating hydrogen to highly reactive radicals, thereby preventing further radical formation (Lapornik *et al*., 2005). The highest abundance of 2-butyl-1-octanol was detected in the sample added with basil essential oil, compared with others. 1-octen-3-ol,1,5-octadien-3-ol and 4-methylcyclohexanone were found only in lipids added with basil essential oil. Only 2-hexyl-1-octanol was detected in the sample containing α-tocopherol. The result confirmed that essential oils were able to retard lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds, which contributed to rancidity in lipids.

4.5.4 Sensory property

Rancid odor of lipids incorporated with lemon essential oil, showing the highest antioxidative activity, and tocopherol at day 0 and 10 are shown in Table 11. Rancid odor score of the control increased markedly at day 10 of storage, whereas the samples added with lemon essential oil and α-tocopherol had lower rancid odor score ($p < 0.05$). Rancid odor score of sample containing antioxidants was around 50% lower than that of the control. There were no differences in rancid odor score amongst all samples at day 0 of storage ($p > 0.05$). The greater development of rancid odor in the control sample correlated well with the higher formation of TBARS (Figure 12B). However, rancid odor score of the sample added with lemon essential oil was not different from that of the sample incorporated with tocopherol ($p > 0.05$). The result suggested that the addition of lemon essential oil could prevent off-odor, mainly rancidity in shrimp hepatopancreas lipid effectively. The efficiency of lemon essential oil was comparable to that of α-tocopherol at the same level used (200 ppm).

4.6 Conclusion

Lipids from hepatopancreas of Pacific white shrimp were susceptible to oxidation during 10 days of storage at 30 ºC. Essential oils used had the impact on oxidative stability differently, in which lemon essential oil showed the highest efficiency in prevention of oxidation. Essential oil from lemon was a potential antioxidant, which exhibited comparable effectiveness to α-tocopherol.

Table 10. Volatile compounds in carotenoid containing lipids in the absence and presence of antioxidant after storage for 10 days at 30 ºC.

Compounds	**Peak area (Abandance) × 10^7**				
	without antioxidant	**α-Tocopherol**	**Lemon essential oil**	**Plai essential oil**	**Basil essential oil**
3-hexanol	38 (18)*	ND	ND	ND	54
2-undecanol	ND (38)	ND	10	ND	ND
2-butyl-1-octanol	104 (65)	ND	33	ND	105
1-octen-3-ol	14 (17)	ND	ND	ND	30
1,5-octadien-3-ol	ND	ND	ND	ND	55
7-ethyl-2-methyl-4-undecanol	32 (32)	ND	ND	ND	ND
2-hexyl-1-octanol	9	6	ND	5	ND
4-terpineol	ND	ND	ND	38	ND
trans-2-Octenol	7 (6)	ND	ND	ND	ND
2-(5-methyl-5-vinyltetrahydro -2-furanyl)propanal	8	ND	ND	ND	ND
2,7-octadien-1-ol	ND (20)	ND	ND	ND	ND
1-hexadecanol	ND	ND	ND	61	ND
4-methylcyclohexanone	ND	ND	ND	ND	40
3-octen-1-ol	8	ND	ND	ND	ND
Methyleugenol	ND	ND	ND	ND	ND
Hexadecanal	15 (7)	ND	ND	ND	ND
2-tert-butyl-4-isopropyl-5 -methylphenol	77 (41)	ND	ND	23	ND

ND: non-detectable

* Value in the parenthesis represent the abundance of compound in sample at 0 day.

Table 11. Rancid odor of carotenoid containing lipid in the absence and presence of essential oil before and after storage for 10 days at 30 ºC.

Samples	**Day**	
	0	**10**
Control	2.54 ± 1.44[‡]B[†]a[*]	12.62 ± 0.57Aa
Tocopherol	2.60 ± 1.44Ba	6.57 ± 1.73Ab
Lemon essential oil	2.50 ± 0.77Ba	6.49 ± 1.84Ab

[‡] Values are given as means ± SD (n=3).

[†] Different uppercase letters in the same row indicate significant differences ($p < 0.05$).

[*] Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

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CHAPTER 5

Oxidative stability of shrimp oil-in-water emulsion as affected by antioxidant incorporation

5.1 Abstract

Shrimp oil is one of the important sources of n-3 fatty acids, which have been known for health benefit. The incorporation of shrimp oil into food emulsion has gained increasing interest. Since shrimp oil is rich in unsaturated fatty acids, it is susceptible to oxidation, leading to development of off-odor and loss in nutritive value. Thus, this study aimed to investigate the oxidative stability of shrimp oil-in-water emulsion (pH 3.6) incorporated with various antioxidants at different levels during the storage at 30 ºC for 12 days. With increasing storage time, progressive formation of hydroperoxide was found in all samples as evidenced by the increase in peroxide values (PV) ($p < 0.05$). The lower PV was found in those added with antioxidants; however PV values varied with different antioxidants added. Sample added with 200 ppm α-tocopherol alone had the higher PV than others ($p < 0.05$). The increase in thiobarbituric acid reactive substances (TBARS) of the control, sample added with 200 ppm α-tocopherol and that containing 500 ppm lecithin were noticeable when storage time increased ($p < 0.05$). Slight increases in TBARS were found in samples added with mixed antioxidants including 50 ppm EDTA + 100 ppm tannic acid, 50 ppm EDTA + 200 ppm α-tocopherol, 50 ppm EDTA + 100 ppm tannic acid + 200 ppm α-tocopherol or 50 ppm EDTA + 100 ppm tannic acid + 500 ppm lecithin + 200 ppm α-tocopherol throughout 12 days of storage ($p < 0.05$). This was concomitant with the decreased formation of volatile compounds and rancid off-odor in emulsion containing mixed antioxidants, especially 50 ppm EDTA + 100 ppm tannic acid and 50 ppm EDTA + 100 ppm tannic acid + 500 ppm lecithin + 200 ppm α-tocopherol. Thus, the use of EDTA in combination with tannic acid could retard the lipid oxidation in the shrimp oil-in-water emulsion more effectively, compared to other combinations of antioxidants tested.

5.2 Introduction

Omega-3 (n-3) polyunsaturated fatty acids are nutritionally important and beneficial for individuals suffering from several diseases, e.g. coronary heart disease, diabetes, and immune response disorders (Djordjevic *et al.*, 2004). Due to health benefits of n-3 fatty acids, the incorporation of lipids rich in those fatty acids into various food products has gained increasing attention (Let *et al.*, 2007). Food emulsions, particularly oil-in-water emulsion such as salad dressings, beverages, etc. consist of small lipid droplets dispersed in an aqueous phase (Grigoriev and Miller, 2009). In general, vegetable oils have been commonly used for most food emulsions. Thus, the use of marine oil, which is high in n-3 fatty acid, can be a means to supplement those essential fatty acids in food emulsion. Nevertheless, the susceptibility to lipid oxidation of emulsions generally limits their shelf-life. Lipid oxidation in emulsions is expected to be initiated at the interface between oil and water (McClements and Decker, 2006). Transition metals, interfacial area, processing conditions, the type of emulsifier, and droplet size etc, can affect the initiation and propagation of oxidation (McClements *et al.*, 2006). To prevent the deteriorative reaction, the antioxidants with capacity of radical scavenging, metal chelation, and oxygen scavenging have been widely used in foods containing lipids (McClements *et al.*, 2006). Some antioxidants can act to prevent lipid oxidation via various modes of action (Mette *et al.*, 2007). Some proteins used as emulsifier are able to alter the properties of emulsion droplet interface in a manner that increases oxidative stability (Hu *et al.*, 2003). Whey protein concentrate exhibited antioxidant properties in emulsion, presumably based on their ability to bind transition metals and scavenge free radicals (Farvin *et al.*, 2010). Furthermore, the polarity and solubility of an antioxidant determine the actual location of antioxidant in food emulsion, which directly influences antioxidative efficacy of antioxidant (Frankel, 1996).

Pacific white shrimp (*Litopenaeus vannamei*) is an important commercial species primarily cultured in Thailand (Nirmal and Benjakul, 2012). In 2012, the production of 540,000 tons of Thai farmed marine shrimp was reported (FAO, 2012). Hepatopancreas, a byproduct generated from the manufacturing of whole shrimp excluding hepatopancreas, has been reported to contain high content of n-3 fatty

acids. Oil from hepatopancreas can be used for making emulsion rich in n-3 fatty acids. Due to the large surface area of oil droplet in emulsion, it is more prone to oxidation, leading to rancidity and unacceptability. Therefore, the use of appropriate antioxidants with multiple modes of actions could prevent lipid oxidation and extend the shelf-life of emulsion rich in n-3 fatty acids effectively. No information regarding the oxidative stability of shrimp oil-in-water emulsion and the impact of antioxidants in emulsion containing shrimp oil has been reported.

5.3 Objective

To investigate the oxidative stability and rancidity of shrimp oil-in-water emulsion incorporated without and with various antioxidants during storage.

5.4 Materials and methods

5.4.1 Chemicals

ρ-anisidine, ammonium thiocyanate, α-tocopherol, L-α-phosphatidylcholine (lecithin) and tannic acid (99.5% purity) were purchased from Sigma (St. Louis. MO, USA). Trichloroacetic acid, anhydrous sodium sulfate, ferrous chloride and ethylenediamine tetraacetic acid (EDTA) were obtained from Merck (Darmstadt, Germany). 2-Thiobarbituric acid and 1,1,3,3-tetramethoxypropane were procured from Fluka (Buchs, Switzerland). Methanol, chloroform, petroleum ether, hydrochloric acid, sulfuric acid and ammonium thiocyanate were purchased from Lab-Scan (Bangkok, Thailand). Whey protein concentrate was obtained from I.P.S. International Co., Ltd. (Bangkok, Thailand).

5.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during November, 2012 and January, 2013. Pooled hepatopancreas (3-5 kg) was placed in polyethylene bag. The bag was imbedded in a

polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18 °C until use, but the storage time was not longer than 1 month. Prior to oil extraction, hepatopancreas was ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec. The moisture content of sample was 70.5% as determined by the AOAC method (AOAC, 2000).

5.4.3 Extraction of oil from hepatopancreas

Oil was extracted from ground hepatopancreas by the Bligh and Dyer method (Bligh and Dyer, 1959). Hepatopancreas (25 g) was homogenized with the mixture of chloroform: methanol: distilled water mixture (50:100:32.4, v/v/v) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 ºC. The homogenate was then added with 50 ml of chloroform and homogenized at 9500 rpm for another 1 min. Thereafter, 25 ml of distilled water were added and the mixture was further homogenized at the same speed for 30 sec. The homogenate was centrifuged at 3000×g at 4 ºC for 15 min and transferred into a separating funnel. The chloroform phase was transferred into the 125 ml Erlenmeyer flask containing 2-5 g of anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper (Whatman International Ltd., Maidstone, England). Chlorofrom was then evaporated at 25 ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan). The residual solvent was removed by nitrogen flushing. Shrimp oil obtained was placed in a vial, flushed with nitrogen gas, sealed tightly and kept at -40 °C until use for emulsion preparation.

5.4.4 Preparation of shrimp oil-in-water emulsion

Shrimp oil-in-water emulsion was prepared following the method of Mette *et al.* (2007) with a slight modification. Whey protein concentrate (2% w/w) and sodium azide (0.02% w/w) were dissolved in 10 mM sodium acetate buffer (pH 3.6). To the solution (90 ml), shrimp oil (10 ml) was gradually added within 1 min,

while homogenization was performed continuously using an IKA Labortechnik homogenizer at a speed of 13,500 rpm for totally 5 min.

To study the effect of antioxidants on storage stability of emulsion, several antioxidants, mixed or alone, were added to obtain different final concentrations. Those included 1) control, 2) lecithin (500 ppm), 3) α-tocopherol (200 ppm), 4) EDTA (50 ppm) + tannic acid (100 ppm), 5) EDTA (50 ppm) + α-tocopherol (200 ppm), 6) EDTA (50 ppm) + tannic acid (100 ppm) + α-tocopherol (200 ppm), 7) EDTA (50 ppm) + tannic acid (100 ppm) + lecithin (500 ppm) + α-tocopherol (200 ppm).

Prior to incorporation, lecithin (500 ppm) and α-tocopherol (200 ppm) were dissolved in shrimp oil, whereas EDTA (50 ppm) and tannic acid (100 ppm) were dissolved in 10 mM sodium acetate buffer (pH 3.6).

5.4.5 Oxidative stability of emulsion added with different antioxidants

Different emulsions containing various antioxidants (14 ml) were transferred into the amber bottle and capped tightly. Sample without antioxidant incorporated was used as the control. The samples were stored at 30 ºC and were taken randomly for analyses at day 0, 2, 4, 6, 8, 10 and 12.

5.4.6 Analyses

5.4.6.1 Peroxide value (PV)

Peroxide value (PV) was determined according to the method of Hu *et al.* (2003) with slight modifications. To 1 ml of emulsion sample, 2 ml of chloroform/methanol (2:1, v/v) were added and mixed using a vortex mixer for 3 s to separate the sample into two phases. The organic solvent phase (20 µl) was mixed with 2.35 ml of chloroform/methanol (2:1, v/v), followed by 50 µl of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v). After 20 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction and expressed as mg cumene hydroperoxide/liter

(l) of emulsion. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

5.4.6.2 Thiobarbituric acid reactive substances (TBARS)

TBARS were determined as described by Buege and Aust (1978). Emulsion sample (0.5 ml) 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 heated in a boiling water (95-100 ºC) for 10 min to develop a pink color, cooled with running tap water and centrifuged at 3600xg at 25 ºC for 20 min using a centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The absorbance of the supernatant was measured 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/l emulsion.

5.4.6.3 Measurement of volatile compounds

The volatile compounds in the control emulsion and those containing different sets of antioxidants rendering high oxidative stability were determined after 12 days of storage using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Iglesias and Medina (2008) with a slight modification. Control emulsion at day 0 was also determined for volatile compounds.

5.4.6.3.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, 1 g of emulsion sample was mixed with 4 ml of deionised water and stirred continuously to disperse the sample. The mixture was heated at 60 ºC in 20 ml headspace vial with equilibrium time of 1 h. The SPME fibre (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was conditioned at 270 ºC 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 ºC for 1 h. The volatile compounds were then desorbed in the GC injector port for 15 min at 270 ºC.

5.4.6.3.2 GC–MS analysis

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). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas, with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

5.4.6.3.3 Analyses of volatile compounds

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). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

5.4.6.4 Sensory evaluation

Before evaluation, the panelists were trained as mentioned in chapter 4. The testing was carried out as described in chapter 4.

5.4.6.5 ζ-Potential measurements

The ζ-potential of the control emulsion (without antioxidants) and the emulsion added with antioxidants yielding the hightest efficacy in prevention of lipid oxidation was determined using a ζ-potential analyser model ZetaPALS (Brookhaven Instruements Co., Holtsville, NY, USA). Prior to analysis, the emulsion was diluted with deionised water to obtain droplet concentration less than 0.02% v/v. ζ -Potential of samples, adjusted to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator model BI-ZTU (Brookhaven Instruments Co., Holtsville, New York, USA), were determined. The values of the zeta potential were calculated by Smoluchowski's formula:

$$\zeta = \frac{4\pi\eta}{\varepsilon}\frac{\upsilon}{U/L}$$

where η and ε are the viscosity and dielectric constant of water, respectively, υ is the mobile velocity of the oil droplets in the electric field, U is the voltage and L is the distance between the two electrodes. The ζ-potential of samples was calculated from the average of ten measurements.

5.4.7 Statistical analysis

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

5.5 Results and discussion

5.5.1 Oxidative stability of shrimp oil-in-water emulsion containing different antioxidants

5.5.1.1 Peroxide value (PV)

Changes in PV of shrimp oil-in-water emulsion without and with several antioxidants incorporated during 12 days of storage are presented in Figure 13A. The control sample and that added with lecithin had the increase in PV within the first 6 days of storage ($p < 0.05$). Subsequently, a slightly decrease in PV was noticeable up to day 10 ($p < 0.05$) and remained constant during 10-12 days ($p > 0.05$). For the sample added with α-tocopherol, PV increased within the first 10 days of storage ($p < 0.05$). The slight decrease in PV was found at day 12 ($p < 0.05$). PV of sample added with combined antioxidants including EDTA + tannic acid, EDTA + α-tocopherol, EDTA + tannic acid + α-tocopherol or EDTA + tannic acid + lecithin + α-tocopherol slightly increased throughout 12 days of storage ($p < 0.05$). The increase in PV of the sample indicated the increasing formation of hydroperoxide, a primary lipid oxidation product. Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (Repetto *et al*., 2012). When comparing PV of all samples, it was found that the sample added with α- tocopherol alone contained the higher PV than others after 2 day of storage ($p < 0.05$). Basically, antioxidant mechanism of tocopherol is hydrogen donation to lipid and/or to peroxide radicals during autoxidation. Thus, they act as chain-breaking antioxidants (Karahadian and Lindsay, 1989). Tocopheroxyl radicals, formed after hydrogen donation, might abstract hydrogen from lipid hydroperoxides or fatty acids. As a consequence, reactive radicals were generated, rather than being scavenged. This result was in agreement with Kim *et al*. (2007) who found that α-tocopherol at the levels of 250, 500, 1000, and 1500 ppm acted as the pro-oxidant in soybean oil during 6 days of storage. Tocopherol, a lipid soluble antioxidant, was found in the hepatopancreas of shrimp (Du *et al*., 2006). Thus, it was more likely that the final concentration of α-tocopherol

in the emulsion was higher than the designated values. Tocopherol at high concentration was reported to promote lipid oxidation (Carocho and Ferreira, 2013).

For other antioxidants used in the present study, they were able to lower the increase in PV in shrimp oil-in-water emulsion, compared with the control ($P < 0.05$). Sample added with lecithin alone showed slightly lower PV than the control after 2 days of storage, indicating its role as antioxidant in the emulsion. Lecithin was found as antioxidant in vegetable oil and fish oil (Chen *et al*., 2011; Judde *et al*., 2003). Phosphate residue might act as the chelator of metal, a pro-oxidant in the system. Lecithin possesses the unique chemical structure containing both lipophilic and hydrophilic groups. The antioxidant activity of lecithin was speculated to be due to their ability to form structures within the lipid phase of the emulsion droplets or to chelate metals (Chen *et al*., 2011). When comparing PV of sample containing EDTA+ α-tocopherol and sample added with α-tocopherol alone, it was found that the sample containing EDTA+ α-tocopherol had the lower PV than those of sample added with α-tocopherol alone throughout 12 days of storage ($p < 0.05$). The result suggested that EDTA alone might have antioxidative activity or exhibited the synergistic effect with α-tocopherol. PV in the sample added with EDTA + α-tocopherol was not different from those found in the sample containing EDTA + tannic acid + α-tocopherol throughout 12 days of storage ($p > 0.05$). EDTA has been known as the potential metal chelator (Djordjevic *et al*., 2004; Wang and Regenstein, 2009). In emulsion with acidic pH, transition metal ions become soluble (Mette *et al*., 2007). In the present study, whey protein concentrate was used as an emulsifier. At pH 3.6 used for emulsion preparation, proteins surrounding oil droplets became positively charged (+ 14 to + 16 mV) as determined by zeta potential analysis. Emulsion had the positive charge throughout storage of 12 days (data not shown). Under such a condition, Fe^{2+} or Fe^{3+} was repelled from oil droplet, thereby preventing the oxidation of polyunsaturated fatty acid in the droplets (Surh *et al*., 2006). The electrical characteriztics of emulsion are important because they determine the droplet stability towards aggregation, as well as their interactions with pro-oxidant cationic transition metal ions (Charoen *et al*., 2011). Thus, whey protein concentrate partially contributed to the oxidative stability of emulsion in conjunction with antioxidants added. It was noted that EDTA scavenged the metal ions in emulsion, thereby

lowering lipid oxidation in emulsion. EDTA has been shown to protect lipid oxidation of foods during storage (Djordjevic *et al*., 2004). In the present study, EDTA in combination with tannic acid and the mixture of EDTA, tannic acid, lecithin and α-tocopherol showed the highest efficacy in retardation of lipid oxidation in emulsion during the storage. It was reported that activity of α-tocopherol could be increased in oil when it was conjugated to the polar head group of lecithin. Lecithin could increase partitioning of the reactive portion of α-tocopherol into the water phase, which could make tocopherol a more efficient free radical scavenger (Laranjinha and Cadenas, 1999). Tannic acid contained a large number of hydrophobic portions, which could align themselves at the oil–water interface and functioned as a hydrogen donor or radical scavenger (Maqsood and Benjakul, 2010). In the present study, tannic acid in conjunction with EDTA was therefore considered as the most potential antioxidants for prevention of lipid oxidation in shrimp oil-in-water emulsion.

5.5.1.2 TBARS

TBARS values of shrimp oil-in-water emulsion without and with added antioxidants incorporated during 12 days of storage are presented in Figure 13B. TBARS levels of the control, sample added with *α*-tocopherol and that containing lecithin increased continuously ($p < 0.05$), and reached the plateau at day 6. After day 8, the marked increases in TBARS were observed up to 12 days ($p < 0.05$), except for that containing tocopherol, which had the constant TBARS during 10-12 days of storage. The increase in TBARS value indicated the formation of the secondary lipid oxidation products (Chaijan *et al*., 2006). TBARS have been used to measure the concentration of relatively polar secondary reaction products, especially aldehydes (Nawar, 1996). The increases in TBARS were coincidental with the decrease in PV, especially with increasing storage time. This was probably due to the destruction of hydroperoxides into the secondary oxidation products, including aldehydes, in the later stages of lipid oxidation (Chaijan *et al*., 2006). Nevertheless, there were no changes in TBARS of sample added with EDTA + tannic acid, EDTA + α-tocopherol, EDTA + tannic acid + α-tocopherol and EDTA + tannic acid + lecithin + α-tocopherol throughout 12 days of storage ($p < 0.05$). The result indicated that

EDTA was essential to prevent the oxidation of shrimp oil-in-water emulsion. Nevertheless, radical scavenger, especially tannic acid, was required to terminate the propagation as evidenced by the negligible change in both PV and TBARS throughout the storage of 12 days.

5.5.1.3 Volatile compounds

Volatile compounds in shrimp oil-in-water emulsion without and with EDTA + tannic acid or EDTA + tannic acid + lecithin + α-tocopherol after 12 days of storage are displayed in Table 12. Volatile compounds in initial emulsion (days 0) was also determined. In general, all compounds detected in emulsion at day 0 were lower in abundance than those found after storage, except for nonanal, which was obtained only in the initial emulsion. The volatile compounds derived from the oxidation of lipids, due to their low perception thresholds, are amongst those chiefly responsible of sensorial properties of products (Montel *et al*., 1996). Shrimp lipids from hepatopancreas contained high content of unsaturated fatty acids and linoleic acid (C18:2(n-6)) was the dominant fatty acid, followed by oleic acid (C18:1(n-9)) (See chapter 2). After storage, the control sample contained several new volatile compounds including 4-ethylcyclohexanol, myrtenol and 4H-pyran-4-one. The higher amount of lipid oxidation products such as benzaldehyde, 3-methoxy-1-(4-methoxyphenyl)-2(E)-propen-1-one, hexadecanal, 2,4-ditert-butylphenol and octadecanal were found in the control sample after 12 days. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acid (Varlet *et al*., 2006). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to follow lipid oxidation in a number of foods. Amongst all the aldehydic compounds, hexadecanal was found to be the major aldehyde in the control sample, followed by octadecanal. After 12 days of storage, it was noted that nonanal was not detected, plausibly due to its decomposition or interaction with other compounds in emulsion. It was postulated that higher lipid oxidation and greater decomposition of hydroperoxide occurred during preparation and storage.

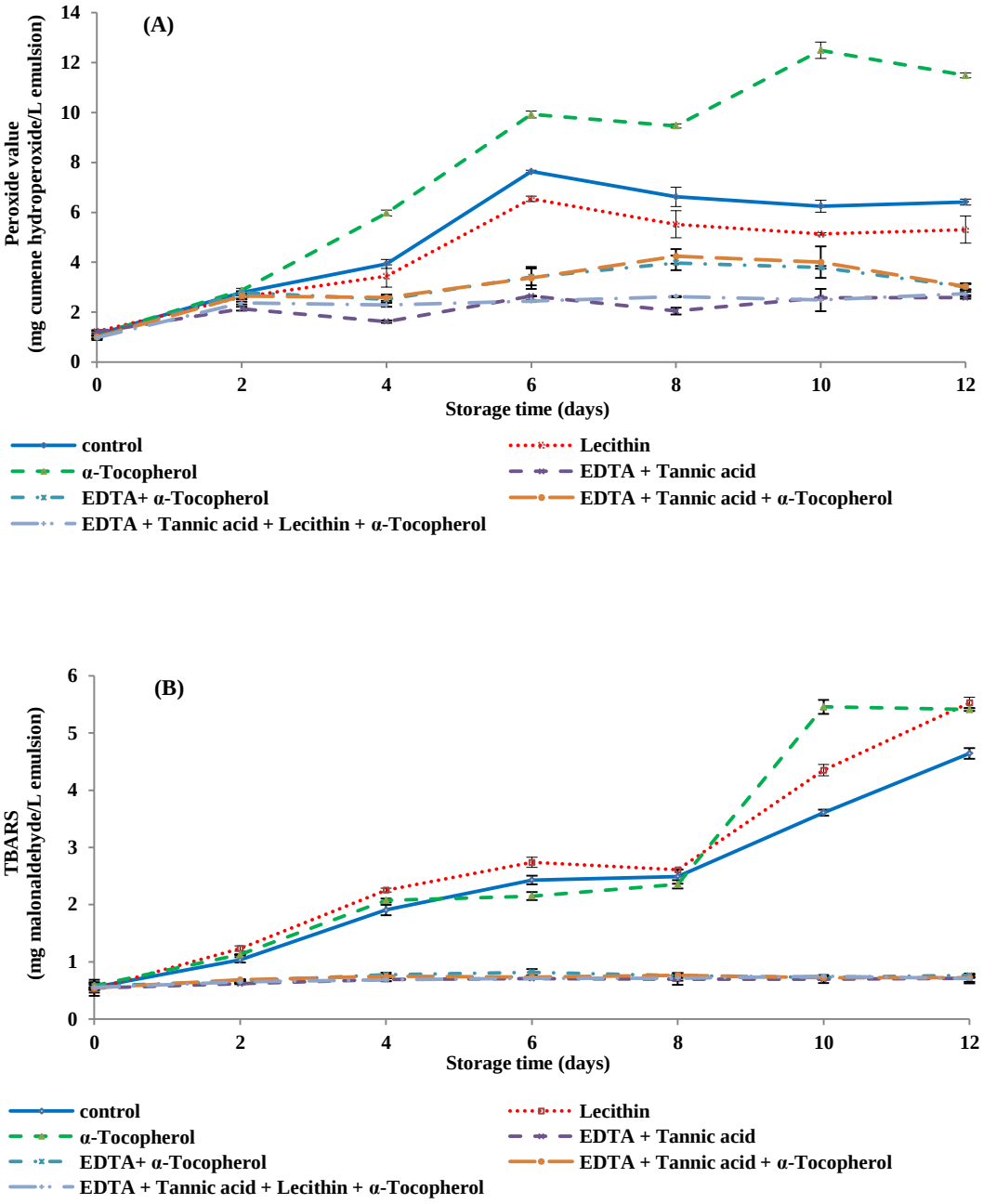

Figure 13 Peroxide values (A) and TBARS values (B) of shrimp oil-in-water emulsion during storage at 30 ºC of 12 days. Bars represent standard deviations (n=3).

Table 12 Volatile compounds in shrimp oil-in-water emulsion in the absence and presence of mixed antioxidants after storage for 12 days at 30 ºC.

Compounds	**Peak area (Abundance) × 10^7**		
	Without antioxidant	**EDTA + Tannic acid**	**EDTA + Tannic acid + Lecithin + α-Tocopherol**
Nonanal	ND (300)*	ND	ND
4-Ethylcyclohexanol	270 (ND)	ND	ND
Benzaldehyde	270 (80)	170	100
Myrtenol	310 (ND)	ND	ND
3-Methoxy-1-(4-methoxyphenyl) -2(E)-propen-1-one	500 (300)	480	410
4H-pyran-4-one	44 (ND)	ND	ND
Hexadecanal	1000 (420)	730	770
2,4-Ditert-butylphenol	190 (120)	200	170
Octadecanal	350 (170)	320	240

ND: non-detectable

* Value in the parenthesis represent the abundance of compound in emulsion at day 0.

However, the formation of volatiles was reduced as the mixed antioxidants were incorporated in shrimp-oil-in-water emulsion. No 4-ethylcyclohexanol, myrtenol and 4H-pyran-4-one were found in emulsion added with mixed antioxidants. Other volatile compounds found in the control were also lower in abundance, compared with those present in the control. The result confirmed that both of EDTA + tannic acid and EDTA + tannic acid + lecithin + α-tocopherol were effective in retarding the lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds, which contributed to rancidity in shrimp oil-in-water emulsion.

5.5.2 Sensory property

Changes in rancidity of shrimp oil-in-water emulsion without and with EDTA + tannic acid or EDTA + tannic acid + lecithin + α-tocopherol are shown in Table 13. There were no differences in rancidity between all samples at day 0 of storage ($p > 0.05$). At day 12, the control sample showed the higher rancidity than other samples ($p < 0.05$). Rancid odor of the sample added with EDTA + tannic acid was not different from that of the sample incorporated with EDTA + tannic acid + lecithin + α-tocopherol ($p > 0.05$). The result was in agreement with the TBARS values (Figure 13B), in which there was no difference between these two samples. The result suggested that the addition of EDTA + tannic acid or EDTA + tannic acid + lecithin + α-tocopherol could prevent off-odor, mainly rancidity in shrimp oil-in-water emulsion effectively. Also, those antioxidants contributed to prevention of nutritional loss and lowering the risk of health problem associated with lipid oxidation (Hayes *et al*., 2011).

5.6 Conclusion

Shrimp oil-in-water emulsion was susceptible to oxidation during 12 days of storage at 30 ºC. Antioxidants used had the impact on oxidative stability differently. Tocopherol alone exhibited prooxidative effect in emulsion during the extended storage. The use of EDTA in combination with tannic acid effectively inhibited lipid oxidation of shrimp oil-in-water emulsion.

Table 13 Rancid odor of shrimp oil-in water emulsion in the absence and presence of mixed antioxidants before and after storage for 12 days at 30 ºC.

Samples	**Day**	
	0	**12**
Control	5.41 ± 1.42[‡] B[†]a[*]	10.78 ± 1.58Aa
EDTA + Tannic acid	5.15 ± 1.05Aa	4.30 ± 1.35Ab
EDTA + Tannic acid + Lecithin + α-Tocopherol	5.18 ± 1.74Aa	4.14 ± 1.63Ab

[‡] Values are given as means ± SD (n=3).

[†] Different uppercase letters in the same row indicate significant differences ($p < 0.05$).

[*] Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

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CHAPTER 6

Micro-encapsulation of Pacific white shrimp oil as affected by emulsification condition

6.1 Abstract

Micro-encapsulation of Pacific white shrimp oil using the mixture of whey protein concentrate and sodium caseinate (1: 1, w/w) as a wall material was carried out. The impact of core/wall material ratios (1: 2 and 1: 4 (w/w)) and homogenizing pressures (2,000 and 4,000 psi) on characteristics and stability of emulsion was investigated. The size of emulsion oil droplets decreased with increasing homogenizing pressure ($p < 0.05$) but was not influenced by core/ wall material ratios ($p > 0.05$). During the extended storage, particle size, flocculation factor (F_f) and coalescence index (C_i) of all emulsions sharply increased, especially in emulsion prepared at 2,000 psi with a core/wall material ratio of 1: 2 ($p < 0.05$). Encapsulation efficiency (EE), particle size and structure of the obtained powders after spray drying were determined. Mean particle sizes (d_{43}) of all powders were larger than those of the emulsion ($p < 0.05$). Similar size (9.14 – 10.18 μm) was obtained for all powders, except that prepared at 2,000 psi with a core/wall material ratio of 1: 2, which had the larger size ($p < 0.05$). Powder prepared using a core/wall material ratio of 1: 4 with homogenizing pressure of 4,000 psi exhibited higher EE (51.3 - 52.8%) than others. Thus, both core/wall material ratio and homogenizing pressure directly affected micro-encapsulation of shrimp oil.

6.2 Introduction

Hepatopancreas, a byproduct generated from the manufacturing of hepatopancreas-free whole shrimp, has been reported to contain high content of n-3 PUFA and astaxanthin (See chapter 2). However, n-3 PUFA are easily oxidized due to their high degree of unsaturation. As a consequence, off-flavor compounds (Kolanowski *et al*., 2007) as well as toxic products (Guillén and Ruiz, 2005) are formed. To lower such a deterioration, encapsulation can be as a key technology in

delaying or inhibiting oxidation and masking undesirable odors and flavors in the final product (Tonon *et al*., 2011). Furthermore, the process converts the oil into a free flowing powder, which can be easily handled and used for nutraceuticals and/or food fortification. Micro-encapsulation can be defined as a process, in which tiny droplets, namely core, are surrounded by a coating of microencapsulating agent. This coating wall can be made of a variety of food grade materials and can protect the entrapped core by providing a physical barrier against environmental conditions (Gallardo *et al*., 2013).

Protein is widely used in the preparation of emulsion and serves as coating wall material during micro-encapsulation process. To ensure the uniform distribution of oil droplet, homogenization with sufficient pressure for emulsification has been widely used in emulsion preparation and encapsulation in the food industry (Ding and Shah, 2009; Schultz *et al*., 2004). The advantage of high pressure homogenization over other technologies is that strong shear and cavitation forces efficiently decrease the diameter of the original droplets (Perrier-Cornet *et al*., 2005). High pressure homogenization induces significant changes in the interfacial protein layer because of the considerable increase in interaction between adsorbed proteins at the interface of the emulsion (Lee *et al*., 2009). Several factors such as process conditions, protein concentration and oil volume fraction have been reported to affect the properties of emulsion (Wang *et al*., 2010). Spray-drying is the most common micro-encapsulation technology used in food industry due to its low cost and available equipment (Gharsallaoui *et al*., 2007). The process involves the atomization of emulsions into a drying medium at a high temperature, resulting in very fast water evaporation. The micro-encapsulated oil was reported to have higher oxidation stability during the extended storage (Calvo *et al*., 2012). Although the micro-encapsulation of several oils or lipids has been reported, no information regarding the micro-encapsulation of shrimp oil has been reported. Due to the differences in composition of oils from different sources, emulsification conditions prior to spray drying can be varied and determine the characteristics of the obtained powder.

6.3 Objective

To investigate the impact of homogenization at varying pressure levels and the ratio of core/wall material on characteristics of encapsulated shrimp oil.

6.4 Materials and methods

6.4.1 Chemicals

Sodium azide (NaN_3) was purchased from Fluka Chemical (Buchs, Switerland). Sodium dodecyl sulphate (SDS) and sodium caseinate were procured from Sigma Chemical Co. (St. Louis, MO, USA). Whey protein concentrate was obtained from I.P.S. International Co., Ltd. (Bangkok, Thailand).

6.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during July and August, 2013. Pooled hepatopancreas (3-5 kg) was placed in a polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18°C until use, but the storage time was not longer than 1 month. Prior to oil extraction, hepatopancreas was thawed using running water (25°C) and ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

6.4.3 Extraction of oil from hepatopancreas

Oil was extracted from hepatopancreas following the method in chapter 3. The prepared hepatopancreas (20 g) was homogenized with 90 ml of cold solvent mixtures (isopropanal: hexane, 50: 50 v/v (4°C)) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4 ºC. The extract was filtered using a Whatman filter paper No.4 (Whatman International Ltd.,

Maidstone, England). The residue was extracted with cold solvent mixtures for another two times. The hexane fractions were pooled and repeatedly washed with an equal quantity of 1% NaCl in order to separate the phases and remove traces of polar solvents. Hexane fraction was then added with 2-5 g of anhydrous sodium sulphate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvent was evaporated at 40 ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. The obtained oil was used for micro-encapsulation.

6.4.4 Characteristics of shrimp oil-in-water emulsion as affected by core/wall material ratio and homogenizing pressure

6.4.4.1 Preparation of shrimp oil-in-water emulsion

Aqueous stock solution of whey protein concentrate and sodium caseinate (1:1 ratio) in deionised water containing 0.03% (w/w) sodium azide was prepared, and stirred overnight using a magnetic stirrer at room temperature (28 – 30°C). The solution obtained was used as 'wall material'. Shrimp oil was added into the solution at different ratios (1:2 and 1:4, core/wall material) The mixtures were homogenized at a speed of 10,000 rpm for 3 min using a homogenizer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia). The coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-5000, Microfluidizer, Newton, MA, USA) at different pressure levels (2,000 and 4,000 psi) for four passes. The emulsions were stored in amber bottles and capped tightly. The samples were stored at room temperature and taken for analyses at day 0, 1, 7 and 14.

6.4.4.1.1 Droplet size

Particle size distribution of emulsions was determined using a ZetaPlus zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). Prior to analysis, emulsion was diluted with 1 % (w/v) sodium dodecyl sulphate (SDS) solution in order to dissociate flocculated droplets. The surface-weighted mean (d_{32}) and the volume-weighted mean particle diameter (d_{43}) of the emulsion droplets were measured as described by Palazolo *et al*. (2011).

6.3.4.1.2 Flocculation and coalescence

To determine flocculation factor (F_f) and coalescence index (C_i), the emulsions were diluted with distilled water in the presence and absence of 1 % (w/v) SDS. F_f and C_i were calculated using the following equations:

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

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

where d_{43+SDS} and $d_{43\text{-}SDS}$ are the volume weight distribution of the emulsion droplets in the presence and absence of 1 % SDS, respectively.

$d_{43+SDS,in}$ is initial value of the volume weight distribution of the emulsion droplets in the presence of 1 % SDS;

$d_{43+SDS,t}$ is the value of the volume weight distribution of the emulsion droplets in the presence of 1 % SDS at the designated storage time.

6.4.4.1.3 ζ-potential

The electrical charge (ζ-potential) of oil droplets in the emulsion was determined using a ZetaPlus zeta potential analyzer at room temperature. The shrimp oil-in-water emulsion was diluted 250-fold prior to measurement. The diluted emulsion was mixed thoroughly and then injected into the measurement chamber of the instrument. The ζ-potential of each individual sample was calculated from the average of five measurements on the diluted emulsion.

6.4.4.2 Preparation of micro-encapsulated oil

Emulsions were subjected to drying using a laboratory scale spray-dryer (LabPlant Ltd., LabPlant SD-05, Huddersfield, UK) with a 1.5 mm diameter nozzle. The emulsion was fed into the main chamber through a peristaltic pump. Feed flow rate was 8.08 ml/min; drying air flow rate was 4.3 m/s and compressor air pressure was 40.61 psi. Air inlet temperature was 180±2°C. The outlet temperature

was controlled at 90±2°C. The obtained powder was transferred into the amber bottle and capped tightly. The samples were drawn randomly for analyses as follows:

6.4.4.2.1 Encapsulation efficiency (EE)

The surface oil was measured by adding 15 ml of hexane to 2 g of powder and shaking with a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY) for 2 min at room temperature. The solvent mixture was then filtered through a Whatman no. 1 filter paper and the collected powder on the filter paper was rinsed three times with 20 ml of hexane (Bae & Lee, 2008). The filtrate solution containing the extracted oil was transferred to a clean flask, which was left to evaporate and then was dried at 60 °C until constant weight was obtained. The surface oil (SO) content was calculated based on the extracted oil (Jafari *et al*., 2008b).

The total oil was determined using the method described by Shahidi and Wanasundara (1995). Five gram of powder was dissolved in 25 ml of a 0.88% (w/v) KCl solution. Then 50 ml of chloroform and 25 ml of methanol were added. The mixture was then homogenized using a high-speed mixer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia) for 5 min at 9,500 rpm. The mixture was transferred to a separation funnel; the chloroform layer was separated and then evaporated using a rotary evaporator at 60 °C to recover the oil. Total oil (TO) content was then calculated.

EE was calculated as follows:

$$\mathrm{EE} = \left(\frac{\mathrm{TO} - \mathrm{SO}}{\mathrm{TO}}\right) \times 100$$

where TO is the total oil content and SO is the surface oil content.

6.4.4.2.2 Powder size

Powder size distribution was measured using a laser light diffraction instrument (Laser Scattering Spectrometer Mastersizer model MAM 5005, Malvern Instruments Ltd., Worcestershire, United Kingdom). The powder sample was

dispersed in 99.5% ethanol and the particle distribution was monitored during five successive readings. The particle size was expressed as the volume-weighted mean particle diameter (d_{43}), which is the mean diameter of a sphere with the same volume, and is generally used to characterize a particle (Frascareli *et al*., 2012).

6.4.4.2.3 Powder morphology

Powder morphology was evaluated by a scanning electron microscopy (SEM). Powder was mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimen was observed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 15 kV with magnifications of ×3000.

6.4.5 Statistical analysis

All experiments were run in triplicate. All analyzes were conducted in five replications. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1960).

6.5 Results and discussion

6.5.1 Characteristics of shrimp oil-in-water emulsion

6.5.1.1 Emulsion droplet size

Particle size of shrimp oil droplet in emulsions containing whey protein concentrate and sodium caseinate (1: 1, w/w) prepared with different ratios of core/wall material (1:2 and 1:4) and homogenizing pressure levels (2,000 and 4,000 psi) expressed as the surface-weighted mean (d_{32}) and the volume-weighted mean particle diameter (d_{43}) was monitored during 14 days of storage at room temperature (Table 14). The d_{32} is directly related to specific surface area. The smaller d_{32} contributes to the higher specific surface area, which offers the increase in protein loads for adsorbing at interface of emulsions (Hebishy *et al*., 2013). The d_{43} can be used as the index of coalescence and flocculation. The increase in d_{43} reflects the

association of individual droplets into larger flocs (Hebishy *et al*., 2013). The d_{32} is more influenced by the small particles, whereas d_{43} is highly influenced by larger ones (Bengtsson and Tornberg, 2011). The emulsion made with core/wall material ratio of 1: 2 and 1: 4 with the same homogenizing pressure had no differences in the d_{32} and d_{43} at 0 day of storage. During the storage, the increases in d_{32} and d_{43} were noticeable in all samples up to day 14 ($p < 0.05$), suggesting the aggregation of droplets. During the storage time, the core/wall material ratio of 1: 4 could retard the increase in particle size in emulsion more effectively than that of 1: 2, regardless of homogenizing pressure used. The result suggested that the high amount of wall material must be sufficient for making the thick and strong film surrounding oil droplets during emulsification.

With the same ratio of core/wall material, the decrease in both d_{32} and d_{43} of droplets was obtained when homogenizing pressure increased ($p < 0.05$). This decrease in oil droplet size might be related to the greater turbulence and shear forces associated with increased homogenizing pressure applied (Hogan *et al*., 2001). Homogenization includes two steps: firstly high shear stress leads to droplet deformation which increases their specific surface area up to disruption. Then the new interface is stabilised by emulsifiers (Floury *et al*., 2000). This process causes the modification of protein conformation, particularly globular protein. Those proteins or peptides with more exposed hydrophobic domains likely adsorbs at increasing droplet interface more effectively. Adsorption of sodium caseinate and whey protein concentrate surrounding interfacial oil droplet provided steric hindrance (Sánchez and Patino, 2005). The reduction of emulsion droplets size, which generally represents an increased stability, results in greater retention of core material (Jafari *et al*., 2008a; Soottitantawat *et al*., 2003). Emulsion droplet size has a pronounced effect on the encapsulation efficiency of core material during spray drying (Soottitantawat *et al*., 2005). The micro-encapsulation of emulsion with small droplet size confers the advantages in terms of emulsion stability, retention of oil in the dried powder and less extractable surface oil (Risch and Reineccius, 1988). During the storage, d_{32} and d_{43} of emulsion prepared using higher pressure (4,000 psi) had the slower rate of increase in size, compared with emulsion prepared using the lower pressure (2,000 psi). The

results indicated that emulsion prepared with higher homogenizing pressure was more stable during the extended storage time.

6.5.1.2 Flocculation and coalescence

Flocculation factor (F_f) and coalescence index (C_i) of shrimp oil-in-water emulsion prepared under different conditions during 14 days of storage at room temperature are shown in Table 15. During storage, all emulsion samples had the increase in F_f, however the rate of change varied. Similar results were found for C_i. Emulsion underwent flocculation when the repulsive forces between the drops were not too strong and if adhesion energy was large enough, the adhesion could be promoted (Langevin *et al*., 2004). Coalescence occurs when two or more oil droplets approach together and join together to form a larger one after the interfacial membrane is ruptured. The process is irreversible and results in the instability of emulsion (Long *et al*., 2012). With the same homogenizing pressure, the emulsion with core/wall material ratio of 1: 2 and 1: 4 had the similar F_f and C_i at day 0 and 1 of storage ($p > 0.05$). After the first day of storage, F_f and C_i increased ($p < 0.05$), especially for emulsion with the core/wall material ratio of 1: 2. The result indicated that the amount of wall material must be enough to stabilise the oil droplets in emulsion.

When comparing F_f and C_i of emulsion with the same core/wall material ratio subjected to different homogenizing pressures, no differences in F_f and C_i were found in emulsion using 2,000 and 4,000 psi at 0 and 1 day of storage ($p > 0.05$). With increasing the storage time, F_f and C_i increased ($p < 0.05$). Such changes were more pronounced in emulsion with homogenizing pressure of 2,000 psi and the core/wall material ratio of 1:2. The result was related well with droplet size of emulsion (Table 14), showing the highest change in d_{32} and d_{43} in emulsion with homogenizing pressure of 2,000 psi with the 1:2 of core/wall material ratio during the storage. Based on F_f and C_i, emulsion with the highest stability could be prepared when homogenizing pressure of 4,000 psi and the core/wall material ratio of 1: 4 were used.

6.5.1.3 ζ-potential

ζ-potential of shrimp oil-in-water emulsion as affected by core/wall material ratio and homogenizing pressures is shown in Table 15. Zeta-potential is the potential difference between the dispersion medium and the stationary layer of fluid attached to the dispersed droplet. This value can be related to the stability of emulsion (Wang *et al*., 2010). All emulsion samples had ζ-potential values higher than -45 mV at 0 day of storage. Negatively charged residues on oil droplet governed by wall material surrounding the shrimp oil droplets mostly contributed to repulsion between droplets, thereby lowering coalescence. At day 0 and 1, no change in ζ-potential was observed ($p > 0.05$). Subsequently, ζ-potential of all samples decreased, especially emulsions with the core/wall material ratio of 1: 2. The layers of protein surrounding droplets might undergo aggregation via ionic interaction during the extended storage as indicated by the change in zeta potential. The insufficient electrostatic repulsion might lead to the development of flocculation and coalescence. Emulsions exhibiting absolute ζ-potential higher than +30 mV or lower than -30 mV tend to be electrostatically stable, while emulsions within the range of (-30) - 30 mV tend to coagulate or flocculate (Wang *et al*., 2011). Emulsion with core/wall material ratio of 1: 2 had low absolute ζ-potential value, regardless of homogenizing pressure. As a result, there was no enough force to prevent the molecules to align together. During the storage, change in ζ-potential of emulsion with the core/wall material ratio of 1: 2 was higher than that of 1: 4 for both homogenizing pressure levels used. The results suggested that the core/wall material ratio of 1: 4 yielded the emulsion with higher stability. In general, high value of ζ-potential means the better stability because of the mutual repulsion between the electrical double layers of macromolecules (Acedo-Carrillo *et al*., 2006). When comparing ζ-potential of all samples with both homogenizing pressure level, no differences in ζ-potential were observed at both core/wall material ratios ($p > 0.05$). Particle size and the stability of emulsion were governed by zeta potential surrounding droplets, which was more likely associated with the charge of protein film at interface.

6.5.2 Characteristics of micro-encapsulated shrimp oil

Emulsions with different processing conditions were subjected to spray drying. The powder containing oil as the core was characterized.

6.5.2.1 Encapsulation efficiency (EE)

EE of micro-encapsulated shrimp oil prepared using the different core/wall material ratio and homogenizing pressures is shown in Table 16. EE reflects the degree of protection afforded by the wall material to oil droplets (Hogan *et al*., 2001). EE varied from a minimum value of 14.65% to a maximum value of 52.05%. EE values from 0% to 95% were reported (Baik *et al*., 2004; Hardas *et al*., 2000; Kha *et al*., 2010; Klinkesorn *et al*., 2006). This was dependent on the type and composition of wall material, the ratio of core/wall material, the drying process used, and the stability and physicochemical properties of the emulsions. The highest EE was found in the sample prepared from emulsion with core/wall material ratio of 1: 4 and homogenizing pressure of 4,000 psi. The surface oil was lower with coincidental increase in EE in sample prepared from emulsion with core/wall material ratio of 1: 4 than those of 1: 2. This result was in agreement with Rodea-González *et al*. (2012) who reported that EE was increased and surface oil decreased when core/wall material increased from 1:2 to 1:3. The core material is embedded as microparticles within the wall material. As the ratio of core/wall material is increased, the ability of the wall material to retain and protect the core material is increased (Hojjati *et al*., 2011), resulting in a lower surface oil and higher EE. The surface oil is critical to encapsulate shrimp oil, since oil on the surface of a particle may readily susceptible to oxidation and the development of rancidity (Quispe-Condori *et al*., 2011). Furthermore, the wettability and dispersability of the powder caused by high surface oil could be lowered (Vega *et al*., 2005).

Table 14 Particle size of droplets in shrimp oil emulsions containing whey protein and sodium caseinate with different core/wall material ratios and homogenizing pressures during the storage.

Core/wall material ratio (w/w)	Pressure used (psi)	Storage time (day)	d_{32} (nm)	d_{43} (nm)
1: 2	2,000	0	169.12 ± 0.51Ad	188.64 ± 1.02Ad
		1	171.64 ± 0.32Ac	192.86 ± 0.22Ac
		7	239.02 ± 1.18Ab	269.47 ± 1.37Ab
		14	334.00 ± 2.42Aa	377.84 ± 2.12Aa
	4,000	0	167.73 ± 0.54Bd	186.00 ± 0.57Bd
		1	171.84 ± 0.70Ac	191.76 ± 0.34Bc
		7	222.29 ± 0.62Bb	247.81 ± 1.24Bb
		14	293.42 ± 2.24Ba	328.26 ± 2.63Ba
1: 4	2,000	0	168.20 ± 0.57Ac	188.54 ± 1.45Ad
		1	170.16 ± 2.55Ac	190.49 ± 0.37Ac
		7	198.61 ± 0.56Ab	223.06 ± 0.87Ab
		14	262.54 ± 1.43Aa	295.85 ± 1.76Aa
	4,000	0	167.09 ± 0.34Bd	185.19 ± 1.34Bd
		1	170.73 ± 1.54Ac	189.76 ± 0.25Bc
		7	193.48 ± 1.24Bb	216.75 ± 1.11Bb
		14	244.35 ± 2.45Ba	273.57 ± 2.68Ba

Data are expressed as mean ± SD (n=5)

Lowercase letters in the same column within the same pressure and core/wall material ratio indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same storage time and core/wall material ratio indicate significant difference ($p < 0.05$).

Table 15 Flocculation, coalescence and ζ –potential of droplets in shrimp oil emulsions containing whey protein and sodium caseinate with different core/wall material ratios and homogenizing pressures during the storage

Core/wall material ratio (w/w)	Pressure used (psi)	Storage time (day)	Flocculation factor (F_f)	Coalescence index (C_i)	ζ -potential (mV)
1: 2	2,000	0	0.98 ± 0.05Ac	-	-46.76 ± 0.75Ac
		1	1.00 ± 0.04Ac	2.24 ± 0.31Ac	-46.71 ± 1.21Ac
		7	1.37 ± 0.02Ab	42.85 ± 2.42Ab	-37.64 ± 2.11Ab
		14	1.93 ± 0.10Aa	100.30 ± 3.46Aa	-31.26 ± 1.07Aa
	4,000	0	0.84 ± 0.10Ac	-	-46.84 ± 1.14Ac
		1	0.87 ± 0.09Ac	2.89 ± 0.31Ac	-46.56 ± 0.73Ac
		7	1.07 ± 0.02Bb	33.23 ± 2.42Bb	-37.15 ± 0.54Ab
		14	1.45 ± 0.10Ba	76.49 ± 3.46Ba	-31.01 ± 0.89Aa
1: 4	2,000	0	0.96 ± 0.05Ac	-	-46.67 ± 0.69Ac
		1	0.97 ± 0.05Ac	2.03 ± 0.71Ac	-46.27 ± 0.93Ac
		7	1.11 ± 0.03Ab	18.30 ± 2.52Ab	-44.66 ± 0.85Ab
		14	1.45 ± 0.03Aa	56.90 ± 3.23Aa	-38.15 ± 0.61Aa
	4,000	0	0.92 ± 0.06Ab	-	-47.83 ± 1.78Ac
		1	0.92 ± 0.02Ab	2.47 ± 0.64Ac	-46.32 ± 1.00Ac
		7	0.94 ± 0.04Bb	17.04 ± 1.55Ab	-43.41 ± 1.19Ab
		14	1.15 ± 0.02Ba	47.72 ± 2.21Ba	-37.94 ± 1.32Aa

Data are expressed as mean ± SD (n=5)

Lowercase letters in the same column within the same pressure and core/wall material ratio indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same storage time and core/wall material ratio indicate significant difference ($p < 0.05$).

The powder made from the emulsion having a core/wall material ratio of 1: 2 and homogenizing pressure of 2,000 and 4,000 psi showed the similar surface oil and EE ($p > 0.05$). For the powder prepared from emulsion with core/wall material ratio of 1: 4, the lower surface oil and higher EE was noticeable when homogenizing pressure of 4,000 psi was implemented, compared with those of 2,000 psi. This result indicated that the emulsification condition (core/wall material ratio and homogenizing pressure level) had the marked influence on encapsulation as well as the characteristic of powder.

6.5.2.2 Powder particle size

Powder particle size of the encapsulated shrimp oil produced with the different core/wall material ratios and homogenizing pressures is shown in Table 16. Powders had the average diameter ranging from 9.14 to 10.18 μm, except the powder prepared from emulsion having core/wall material ratio of 1: 2 and homogenizing pressure of 2,000 psi, which the diameter of 13.05 μm. Homogenizing pressure had had no effect on size of powder when core/wall material ratio of 1: 4 was used. The result indicated that core/wall material ratio of 1: 4 was sufficient to align themselves surrounding shrimp oil droplets prior to spray drying. However, there was difference in EE between both homogenizing pressures. Thus, higher pressure more likely dissociated the oil droplets, in which wall material could occupy rapidly and acted as wall of droplets. This was evidenced by high EE when high homogenizing pressure (4,000 psi) was used. Finney *et al*., (2002) and Jafari *et al*., (2008a) reported that particle size had no impact on retention of some volatile and oil, as are solids in emulsion was sufficiently high. When comparing the size of shrimp oil emulsions and powder, an increase in powder particle size was observed after spray drying process. The increased powder size partly arose from coalescence of oil droplet in emulsion during the spray drying process (Drusch *et al*., 2007). Additionally, the wall generated also contributed to the increased diameter. The diameter of the microcapsules depends on homogenization parameters, the materials used and conditions of the spray drying process (Kolanowski *et al*., 2006).

Table 16 Powder size, surface oil, total oil and encapsulation efficiency of different encapsulated shrimp oil powder prepared from emulsions containing whey protein and sodium caseinate with different core/wall material ratios and homogenizing pressures

Core/wall material ratio (w/w)	Pressure used (psi)	Powder size ($d_{4,3}$,µm)	Surface oil (%w/w)	Encapsulation efficiency (%)
1: 2	2,000	13.05 ± 1.12aA	23.86 ± 0.47aA	14.65 ± 1.38aB
	4,000	9.74 ± 0.32bA	23.51 ± 1.53aA	14.96 ± 0.39aB
1: 4	2,000	9.55 ± 0.27aB	12.42 ± 0.28aB	31.50 ± 1.81bA
	4,000	9.66 ± 0.52aA	9.01 ± 1.23bB	52.05 ± 0.71aA

Data are expressed as mean ± SD (n=3)

Lowercase letters in the same column within the same core/wall material ratio indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same pressure indicate significant difference ($p < 0.05$).

6.5.2.3 Powder morphology

SEM microphotographs of the powders produced from emulsion with the different core/wall material ratios and homogenizing pressures are shown in Figure 14. All powders exhibited similar surface morphologies. Powders prepared from emulsions with core/wall material ratio of 1: 2 using both homogenizing pressure levels appeared to be agglomerated. Clumps of particles were observed (Figure 14A and 14B). This was plausibly due to high surface oil levels (Table 16), which made the particles stick together. For powders prepared from emulsion with core/wall material ratio of 1: 4 with both homogenizing pressure levels (Figure 14C and 14D), a higher level of surface indentation was obtained, compared with those prepared from emulsion with a core/wall material ratio of 1: 2. The different morphologies and surface irregularities were governed by composition of the

emulsion, droplet size, and temperature during the drying process (Handscomb and Kraft, 2010). Wrinkles or dimples on the surface were observed in all powders. These results are consistent with Klinkesorn *et al*., (2006) and Ahn *et al*., (2008), who detected wrinkles on the surface of the particles in spray-dried powder. Wrinkles were attributed to the results of mechanical stresses induced by uneven drying at different parts of the droplets during the early stages of drying (Sheu and Rosenberg, 1998), to the movement of the moisture during the surface drying period (Walton, 2000), and to the effect of a surface tension-driven viscous flow (Sheu *et al*., 1998). Wrinkles of the particle followed by an incipient expansion may induce changes in the size of particles and causes the broken wall material (Alamilla-Beltrán *et al*., 2005).

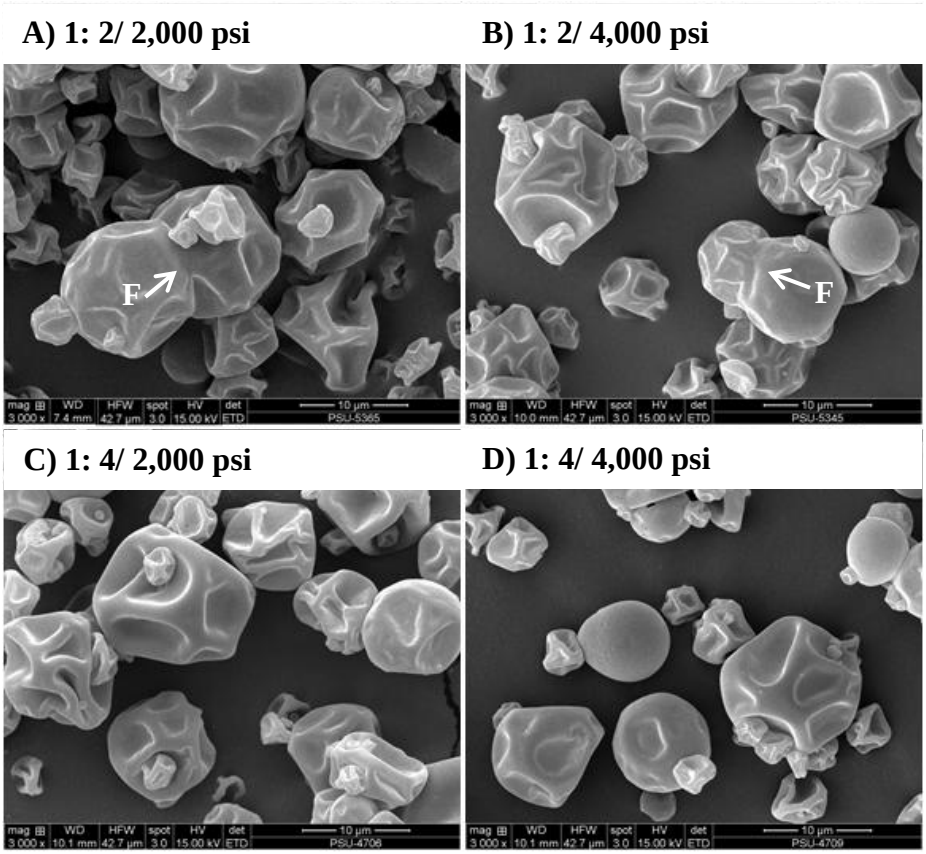

Figure 14 Surface morphology of micro-encapsulated shrimp oil prepared from emulsion containing a mixture of whey protein concentrate and sodium caseinate (1: 1, w/w) with different core/wall material ratios and homogenizing pressures. (Magnification: ×3000). F = fused together.

6.6 Conclusion

Shrimp oil was encapsulated from emulsion stabilised by whey protein concentrate and sodium caseinate (1: 1, w/w). Core/wall material ratio and homogenizing pressure directly had the impact on emulsion and resulting encapsulated powder. Higher homogenizing pressure reduced droplet size of emulsion. Emulsification at 4,000 psi with a core/wall material ratio of 1: 4 yielded the emulsion with the highest stability during 14 days of storage. After spray drying, emulsion with high core/wall material ratio and homogenizing pressure rendered the micro-encapsulated shrimp oil powder with higher EE. Thus, shrimp oil could be encapsulated using a mixture of whey protein concentrate and sodium caseinate (1: 1, w/w) as encapsulating agents with core/wall material ratio of 1: 4 and homogenizing pressure level of 4,000 psi, followed by spray-drying. However, the improvement of encapsulation process for shrimp oil is still required.

6.7 References

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CHAPTER 7

Impact of wall materials and antioxidants on encapsulation efficiency and oxidative stability of micro-encapsulated shrimp oil

7.1 Abstract

The effect of different wall materials including whey protein concentrate (WPC): sodium caseinate (SC) (1:1, w/w), WPC: SC: gum arabic (1: 1: 2, w/w/w), WPC: SC: glucose syrup (1: 1: 2, w/w/w) and WPC: SC: maltodextrin (1: 1: 2, w/w/w) on micro-encapsulation of shrimp oil was investigated. The highest encapsulation efficiency (EE) was obtained when the mixture of WPC, SC and glucose syrup was used (84.43 – 88.19%), while the lowest EE was obtained for the sample using WPC and SC (50.37 – 53.05 %). All powders had low moisture contents (< 3.5%) and no difference in volume weighted mean particle diameter (d_{43}) was found ($p > 0.05$). Oxidative stability of shrimp oil microcapsules using the mixture of WPC, SC and glucose syrup as wall materials incorporated with different antioxidants was examined during storage at 30 ºC for 6 weeks. With increasing storage time, the increase in peroxide values (PV) was observed ($p < 0.05$). The lowest PV was found in those added with lemon essential oil + EDTA + tannic acid after 6 weeks of storage ($p < 0.05$). Thus, the use of appropriate wall materials in combination with selected antioxidants yielded micro-encapsulated shrimp oil with high oxidative stability.

7.2 Introduction

Hepatopancreas, a byproduct generated from the manufacturing of hepatopancreas-free whole shrimp, is the excellent source of oil rich in polyunsaturated fatty acids (PUFA) and astaxanthin (See chapter 2). Apart from their nutritive value, their consumption may have the beneficial effects on health (Carneiro *et al*., 2013). Nevertheless, one of the major problems associated with oils rich in PUFAs is their high susceptibility to oxidative deterioration and the formation of undesirable flavor, especially when exposed to oxygen, light and moisture (Kolanowski *et al*., 2007). To prevent such a deterioration, several technologies have

been implemented, such as packaging (Raei and Jafari, 2013) the use of antioxidant (Chang *et al*., 2013), etc.

Encapsulation can be as a key technology in delaying or inhibiting oxidation and masking undesirable odors and flavors in the final product (Tonon *et al*., 2011). The "wall" formed by the microencapsulating agent is designed to protect the core from deterioration and release it under the desired conditions (Young *et al*., 1993). Selecting the most appropriate wall material is an important step in developing micro-encapsulation (Calvo *et al*., 2010). The use of mixed wall materials could increase the encapsulation efficiency (EE) and shelf-life of core (Jimenez *et al*., 2006; Pérez-Alonso *et al*., 2008). Wall material could act as a barrier, protecting the core from oxygen and light or avoiding the contact with other ingredients. Wall materials for microencapsulation of oil must have high stability, high water solubility, and a tendency to form a fine and dense network during drying (Calvo *et al*., 2012). Wall material possessing emulsifying properties should not permit lipid separation from the emulsion during dehydration (Bae and Lee, 2008; Gharsallaoui *et al*., 2007). Proteins and carbohydrates are frequently studied as matrices to encapsulate lipophilic compounds by spray drying (Charve and Reineccius, 2009; Gharsallaoui *et al*., 2007). Spray-drying is the most common micro-encapsulation technology used in food industry due to its low cost, continuous production, easiness of industrialization and available equipment (Gharsallaoui *et al*., 2007).

Although encapsulation itself prevents lipid oxidation, antioxidants is required to ensure maximum protection during processing and storage of microencapsulated oils (Serfert *et al*., 2009). To enhance the efficacy in prevention of lipid oxidation, the use of combined antioxidants with different modes of action could be a means to stabilize the core. Primary antioxidants with radical scavenging have been widely used in foods, particularly those from natural sources. Tannic acid has been reported to possess antioxidative activity (Maqsood and Benjakul, 2010). Essential oils have been reported to have antioxidative activity (Özcan and Arslan, 2011). The use of mixed antioxidants could prevent lipid oxidation and extend the shelf-life of encapsulated shrimp oil rich in PUFA effectively.

7.3 Objective

To study the influence of mixed wall materials on EE and the impact of antioxidants on oxidative stability of encapsulated shrimp.

7.4 Materials and methods

7.4.1 Chemicals

Sodium azide (NaN_3) was purchased from Fluka Chemical (Buchs, Switerland). Ethylenediamine tetraacetic acid (EDTA) was obtained from Merck (Darmstadt, Germany). Tannic acid (99.5% purity) was purchased from Sigma (St. Louis. MO, USA). Sodium caseinate (SC) was procured from Vicchi enterprise Co., Ltd. (Bangkok, Thailand). Whey protein concentrate (WPC) was obtained from *I.P.S. International* Co., Ltd. (Bangkok, Thailand). Maltodextrin (Dextrose equivalent; 10) was purchased from Nutrition Sc Co., Ltd. (Bangkok, Thailand). Gum Arabic was purchased from OV chemical and supplies Co., Ltd. (Chiang Mai, Thailand). Glucose syrup (Dextrose equivalent; 40-43) was obtained from Charoenworrakit Co., Ltd. (Samut Prakan, Thailand). Essential oils (100% purity) from lemon, plai and basil were obtained from Botanicessence (Bangkok, Thailand).

7.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during November and December, 2013. Pooled hepatopancreas (3-5 kg) was placed in a polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18°C until use, but the storage time was not longer than 1 month. Prior to oil extraction, hepatopancreas was thawed using running water (25°C) and ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

7.4.3 Extraction of oil from hepatopancreas

Oil was extracted from hepatopancreas following the method in chapter 3. The prepared hepatopancreas (20 g) was homogenized with 90 ml of cold solvent mixtures (isopropanal: hexane, 50: 50, v/v) (4°C) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4ºC. The extract was filtered using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, England). The residue was extracted with cold solvent mixtures for another two times. The hexane fraction was pooled and repeatedly washed with an equal quantity of 1% NaCl in order to separate the phases and remove traces of polar solvents. Hexane fraction (approximately 135 ml) was then added with 2-5 g anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvent was evaporated at 40ºC using an EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. The obtained oil was used for micro-encapsulation.

7.4.4 Preparation of shrimp oil-in-water emulsion

Aqueous stock mixed solution of WPC, SC and different carbohydrates (gum arabic, maltodextrin and glucose syrup) at a ratio of 1: 1: 2 (w/w/w) in deionised water was prepared. The mixture was stirred overnight using a magnetic stirrer at room temperature (28 – 30°C). The solution containing WPC and SC at a ratio of 1: 1 (w/w) was used as the control. All solutions obtained were used as ‘wall material’. Shrimp oil was added into the solution at a core/wall material ratio of 1:4. The mixtures were homogenized at a speed of 10,000 rpm for 3 min using a homogenizer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia). The obtained coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-5000, Microfluidizer, Newton, MA, USA) at a pressure level of 4,000 psi for four passes.

7.4.5 Preparation of micro-encapsulated shrimp oil

Emulsions were subjected to drying using a laboratory scale spray-dryer (LabPlant Ltd., LabPlant SD-06A, Huddersfield, UK) with a 0.5 mm diameter

nozzle. The emulsion was fed into the main chamber (215 mm OD × 500 mm long) through a peristaltic pump. Feed flow rate was 8.08 ml/min; drying air flow rate was 4.3 m/s and compressor air pressure was 40.61 psi. Air inlet temperature was 180±2°C. The outlet temperature was controlled at 90±2°C. The obtained powder was transferred into the amber bottle and capped tightly. The samples were taken randomly for analyses as follows:

7.4.5.1 Encapsulation efficiency (EE)

Surface oil (SO) of powder was measured by adding 15 ml of hexane to 2 g of powder and shaking with a vortex mixer (G-560E, Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY) for 2 min at room temperature. The solvent mixture was then filtered through a Whatman no. 1 filter paper and the collected powder on the filter paper was rinsed three times with 20 ml of hexane (Bae and Lee, 2008). The filtrate solution containing the extracted oil was transferred to a clean flask, which was left to evaporate and then was dried at 60 °C until constant weight was obtained. The SO content was calculated based on the extracted oil (Jafari *et al*., 2008b).

Total oil (TO) was determined using the method described by Shahidi and Wanasundara (1995). Five gram of powder was dissolved in 25 ml of 0.88% (w/v) KCl solution. Then 50 ml of chloroform and 25 ml of methanol were added. The mixture was then homogenized using homogenizer a high-speed mixer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia) for 5 min at 9,500 rpm. The mixture was transferred to a separation funnel; the chloroform layer was separated and then evaporated using a rotary evaporator at 60 °C to recover the oil. The TO content was then calculated.

EE was calculated as follows:

$$\mathrm{EE} = \left(\frac{\mathrm{TO} - \mathrm{SO}}{\mathrm{TO}}\right) \times 100$$

where TO is the total oil content and SO is the surface oil content.

7.4.5.2 Powder size

Powder size distribution was measured using a laser light diffraction instrument (Laser Scattering Spectrometer Mastersizer model MAM 5005, Malvern Instruments Ltd., Worcestershire, United Kingdom). The powder sample was dispersed in 99.5% ethanol and the particle distribution was monitored during five successive readings. The particle size was expressed as the volume-weighted mean particle diameter (d_{43}), which is the mean diameter of a sphere with the same volume, and is generally used to characterize a particle (Frascareli *et al*., 2012).

7.4.5.3 Powder morphology

Powder morphology was evaluated by a scanning electron microscopy (SEM). Powder was mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimen was observed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 15 kV with magnifications of ×3000.

7.4.5.4 Moisture content

Moisture content was determined as per the oven method (AOAC, 2000).

7.4.6 Oxidative stability of encapsulated shrimp oil added with different antioxidants

Emulsion containing the selected wall material yielding the highest EE was added with different antioxidants including 1) lemon essential oil 2) tannic acid + EDTA and 3) lemon essential oil, tannic acid + EDTA. Sample without antioxidant incorporated was used as the control. Prior to the incorporation, lemon essential oil (200 ppm) was dissolved in shrimp oil, whereas EDTA (50 ppm) and tannic acid (100 ppm) were dissolved in aqueous stock solution. Emulsion and micro-encapsulated shrimp oil were prepared in the same manner as previously described. The obtained powder was stored in the amber bottle and capped tightly at 30 ± 1 ºC and taken for

analyses at week 0, 1, 2, 4 and 6. At the time designated, powders were subjected to analyses.

7.4.6.1 Peroxide value (PV)

PV was determined according to the method of Hu *et al*. (2003) with slight modifications. The powder (0.2 g) was dissolved in 5 ml of distilled water. Then the mixture was stirred for 5 min to allow complete dispersion. To 1 ml of the mixture, 2 ml of chloroform/methanol (2:1, v/v) were added and mixed using a vortex mixer for 3 sec to separate the sample into two phases. The organic solvent phase (50 µl) was mixed with 2.35 ml of chloroform/methanol (2:1, v/v), followed by 50 µl of 30% ammonium thiocyanate (w/v) and 50 µl of 20 mM ferrous chloride solution in 3.5% HCl (w/v). After 20 min, the absorbance of the colored solution was read at 500 nm using a spectrophotometer (Shimadzu, Kyoto, Japan). Blank was prepared in the same manner, except the distilled water was used instead of ferrous chloride. PV was calculated after blank substraction and expressed as mg cumene hydroperoxide/g oil. A standard curve was prepared using cumene hydroperoxide with the concentration range of 0.5–2 ppm.

7.4.6.2 Volatile compounds

The volatile compounds in all powder samples were determined after 6 weeks of storage using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Iglesias and Medina (2008) with a slight modification. The control powder at day 0 was also determined for volatile compounds.

7.4.6.2.1 Extraction of volatile compounds by SPME fibre

To extract volatile compounds, Powder (1 g) was mixed with 4 ml of deionised water and stirred continuously to disperse the sample. The vial was tightly closed by means of a capper. A carboxen–polydimethylsiloxane solid phase micro-extraction fiber (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was used to adsorb the volatile lipid oxidation compounds

released from the sample. The fibre was inserted into the vial and equilibrated at 40 °C for 30 min prior to GC–MS analysis.

7.4.6.2.2 GC–MS analysis

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). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

7.4.6.2.3 Analyses of volatile compounds

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). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

7.4.7 Statistical analysis

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

7.5 Results and discussion

7.5.1 Characteristics of micro-encapsulated shrimp oil

7.5.1.1 Moisture content

Moisture contents of the micro-encapsulated shrimp oil having different wall materials are presented in Table 17. Moisture contents of all samples were in the range of 1.12-3.28%. The micro-encapsulated shrimp oil produced from the mixture of WPC, SC and glucose syrup (1: 1: 2, w/w/w) showed the lower moisture content than others ($p < 0.05$). In general, samples showed the differences in moisture content when various wall materials were used. Different wall materials can exhibit the different crust formation. As a result, water diffusion through the wall could be varied, thus affecting the water evaporation and rendering the powders with different moisture content (Frascareli *et al*., 2012). Şahin-Nadeem *et al*., (2013) reported that the change in moisture content of the sage powders depended on the type and concentration of wall materials. Moisture content is important factor affecting storage stability of powders (Guadarrama-Lezama *et al*., 2012). Water in powder was readily available for hydrolytic and enzymatic reactions. At low water content, the degradation of the micro-encapsulated oil was low (Lim *et al*., 2012). However, food dried to less than 2-3% moisture content become susceptible to lipid oxidation (Labuza and Dugan Jr, 1971). Reineccius (2004) reported that when moisture content reaches values lower than 7%, water diffusion through the food matrix decreases. As a consequence, the effect of moisture content on the physical and chemical characteristics of micro-encapsulated oils and the accessibility of oxygen to oil through the porous of wall material were lowered.

7.5.1.2 Encapsulation efficiency (EE)

EE of micro-encapsulated shrimp oil obtained from emulsions produced with different wall materials is shown in Table 17. EE reflects the degree of protection afforded by the wall material to oil droplets (Hogan *et al*., 2001). EE of all samples varied from 50.37 to 88.19% ($p < 0.05$). The results indicated that the use of protein combination with glucose syrup as the wall material could improve EE of micro-encapsulated shrimp oil more effectively than protein alone and protein in combination with gum arabic or maltodextrin ($p < 0.05$). EE was therefore influenced by the type and composition of wall materials Carneiro *et al.* (2013). The highest EE observed for microcapsules prepared using WPC: SC: glucose syrup (1: 1: 2, w/w/w) as wall materials might be associated with hydrophilic nature of the wall material, which would limit the accessibility of hydrophobic core materials (Hogan *et al*., 2001). Sugar such as glucose reported to crystallize, causing the powders to collapse (Harnkarnsujarit and Charoenrein, 2011). Collapse of spray-dried powders is linked to a decrease in porosity. Thus, lipid oxidation could be lower due to a decrease in the diffusivity of O_2 into the core (Thomas *et al*., 2004). Gharsallaoui *et al*. (2007) reported that lactose in its amorphous state acted as a hydrophilic sealant that significantly limited the diffusion of the hydrophobic core through the wall material, thus leading to high EE values. The incorporation of carbohydrates into the wall material systems has been shown to improve drying properties of the wall material, probably by enhancing the formation of a dry crust around the drying droplets (Calvo *et al*., 2012). Although carbohydrates have severed desirable properties are an encapsulating agent, such as low viscosities at high solid contents and good solubility, they show low interfacial properties required for high EE (Gharsallaoui *et al*., 2007). The higher EE was coincidental with the decrease in surface oil of the sample. The surface oil represented non-encapsulated oil and has been used as an important parameter determining the product quality. Non-encapsulated oil is prone to oxidation, thus leading to the development of off-flavors and lower acceptability of the product (Drusch and Berg, 2008). In general, wall materials for micro-encapsulation of oil must have emulsifying properties, high water solubility, low

viscosity, and drying properties (Bae *et al*., 2008; Calvo *et al*., 2010). Thus, wall materials were the important factor determining EE for shrimp oil encapsulation.

7.5.1.3 Particle size and morphology of micro-encapsulated shrimp oil

Particle size of the micro-encapsulated shrimp oil using different wall materials expressed as the volume-weighted mean particle diameter (d_{43}). The mean particle size is an important factor in terms of quality and the application of the micro-encapsulation (Guadarrama-Lezama *et al*., 2012). Micro-encapsulated shrimp oil had the average diameter ranging from 10.04 to 10.51 μm (Table 17). There was no difference in d_{43} for all microcapsules using varying wall materials ($p > 0.05$). McNamee *et al*. (1998) reported that particle size of micro-encapsulation is influenced by the nozzle, feed rate, atomizing conditions, air pressure and total solid content. In this study, all the emulsions were spray-dried under the identical operational conditions. As a result, no difference was found in particle size of micro-encapsulated shrimp oil, regardless of wall materials used ($p > 0.05$).

SEM micrographs of the microcapsules powders produced from emulsion with different wall materials are shown in Figure 15. All powders exhibit well-separated micro-encapsules and free of cracks. Powders prepared from emulsion using the mixture of WPC, SC and glucose syrup (Figure 15C) more likely showed the spherical shape and smoother surface than those prepared from emulsions using other wall materials. Glucose syrup containing a greater amount of low-molecular-weight sugar plausibly acted as a plasticizer, preventing irregular shrinkage of the surface during drying. According to Zhang *et al*., (2000), plasticiser is important for the formation of spherical microcapsules with smooth surface. However, some powders showed the shrinkage with non-uniform surface. The spherical and smooth surface generally shows the suitability of the wall materials for encapsulation (Krishnan *et al*., 2005). For other powders (Figure 15A, B and D), a higher level of surface indentation was observed. Surface dents were found on the powders. The composition of feed, nature of the biopolymers used as encapsulating agents, atomization, and temperature during the drying and cooling process played a role in

Table 17 Powder size, surface oil, encapsulation efficiency and moisture content of different encapsulated shrimp oil powder using different wall materials.

Wall materials	**Powder size ($d_{4,3}$, µm)**	**Surface oil (%w/w)**	**Encapsulation efficiency (%)**	**Moisture content (%)**
WPC :SC (1 :1, w/w)	10.09 ± 0.71a	9.00 ± 0.38a	51.71 ± 1.34c	3.10 ± 0.18a
WPC : SC: gum Arabic (1 :1 :2, w/w/w)	10.32 ± 0.59a	8.84 ± 0.14a	51.78 ± 1.30c	1.43 ± 0.26b
WPC : SC: glucose syrup (1 :1 :2, w/w/w)	10.91 ± 0.60a	2.48 ± 0.24c	86.31 ± 1.88a	1.06 ± 0.04c
WPC : SC: maltodextrin (1 :1 :2, w/w/w)	10.04 ± 0.75a	7.74 ± 0.24b	59.81 ± 0.16b	1.35 ± 0.22b

Data are expressed as mean ± SD (n=3).

Different letters within the same column denote significant differences ($p < 0.05$).

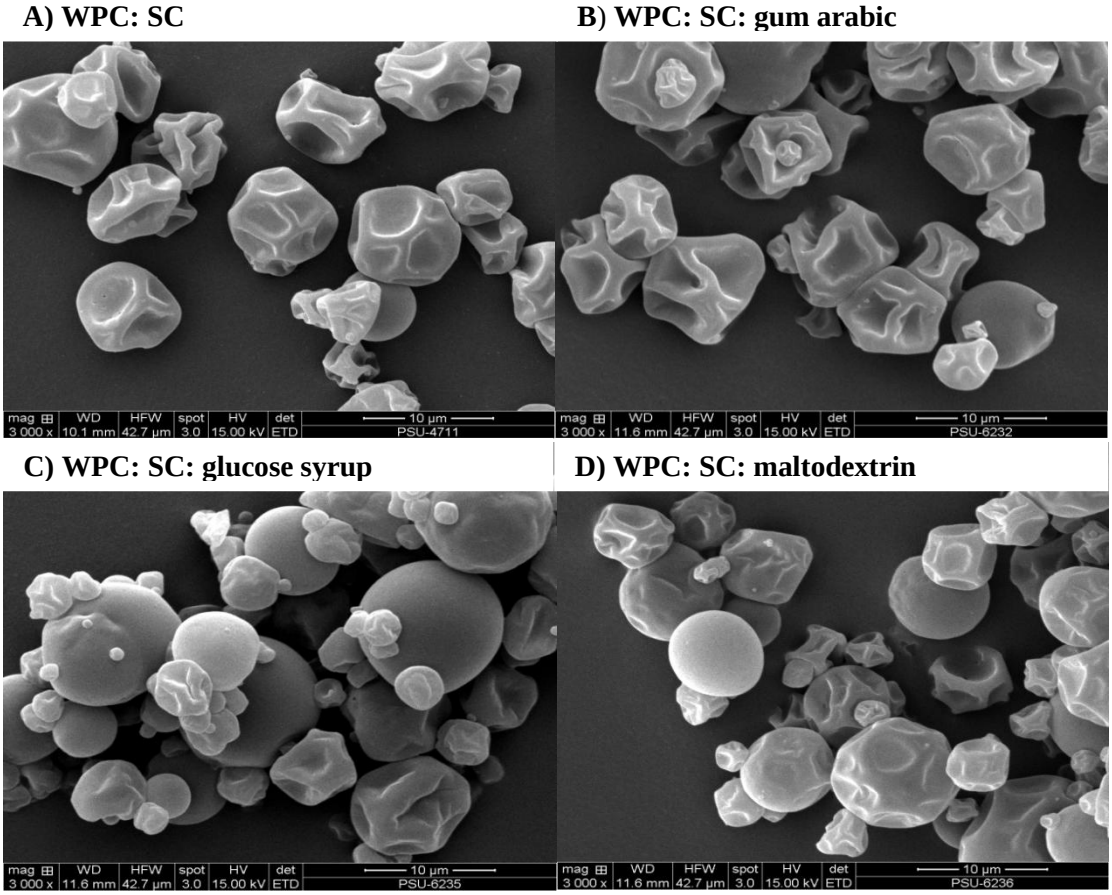

Figure 15 Surface morphology of micro-encapsulated shrimp oil prepared from emulsion containing different wall material. (Magnification: ×3000).

the formation of surface dents (Bylaitë *et al*., 2001; Handscomb and Kraft, 2010). High drying rates usually lead to more rapid wall solidification with consequent formation of less smooth surface (Sheu and Rosenberg, 1998).

7.5.2 Oxidative stability of micro-encapsulated shrimp oil

Microcapsules using the mixture of WPC, SC and glucose syrup were prepared in the presence of different antioxidants. Lipid oxidation of all powders was monitored during the storage of 6 weeks at 30°C.

7.5.2.1 Peroxide value (PV)

Changes in PV of micro-encapsulated shrimp oil without and with different antioxidants incorporated during 6 weeks of storage are presented in Figure

16. All powders showed a low PV at week 0. During the storage, PV in the control powder increased to a higher extent, compared with those incorporated with antioxidant ($p < 0.05$). For PV of powders added with lemon essential oil, EDTA + tannic acid, or lemon essential oil + EDTA + tannic acid remained constant within the first week of storage ($p > 0.05$). Subsequently, an increase in PV was noticeable up to week 6 ($p < 0.05$). With increasing storage time, shrimp oil could be released to the surface of the powders. Those oils could be more prone to oxidation. It could be related to the physical and chemical change of the wall material, which could enhance and the diffusion of oil through the wall. Simultaneously, oxygen could permeate the sample the wall material to the lipid core. As a result, the oxidation of lipid increased accordingly (Aghbashlo *et al*., 2012). Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical, resulting in the oxidative damage of polyunsaturated fatty acids (Repetto *et al*., 2012). The oxidation of oil creates a variety of compounds including free radicals and hydroperoxides (Firestone, 1998). PV is a measure of the amount of hydroperoxide, representing the initial stage of fat and oil deterioration, and is a standard index to monitor food safety and quality (Wang *et al*., 2011).

When comparing PV of all powder samples, it was found that the control powder without antioxidant had higher PV than other powders after 2 weeks of storage, following by those added with lemon essential oil, respectively ($p < 0.05$). It was noted that PV in the powder added with EDTA + tannic acid was not different from those found in the powder containing lemon essential oil + EDTA + tannic acid throughout 4 weeks of storage ($p > 0.05$). Nevertheless, the powder added with lemon essential oil + EDTA + tannic acid showed the lowest PV, compared with other samples after 6 weeks of storage ($p < 0.05$). EDTA has been known as the potential metal chelator (Wang and Regenstein, 2009). Tannic acid contained a large number of hydrophobic portions and functioned as a potential hydrogen donor or radical scavenger (Maqsood *et al*., 2010). In the previous study, EDTA in combination with tannic acid showed the highest efficacy in retardation of lipid oxidation of shrimp oil-in-water emulsion during the storage at 30 °C (Takeungwongtrakul and Benjakul, 2013). In addition, essential oils are composed of complex mixtures of monoterpenes, biogenetically related phenols, and sesquiterpenes (Isman *et al*., 2007). Lemon

essential oils have been reported to possess antioxidative activity and reduce the oxidation (Singh *et al.*, 2012). The result indicated that EDTA, tanic acid and lemon essential oil more likely exhibited the combined effect on the prevention of oxidation of micro-encapsulated shrimp oil.

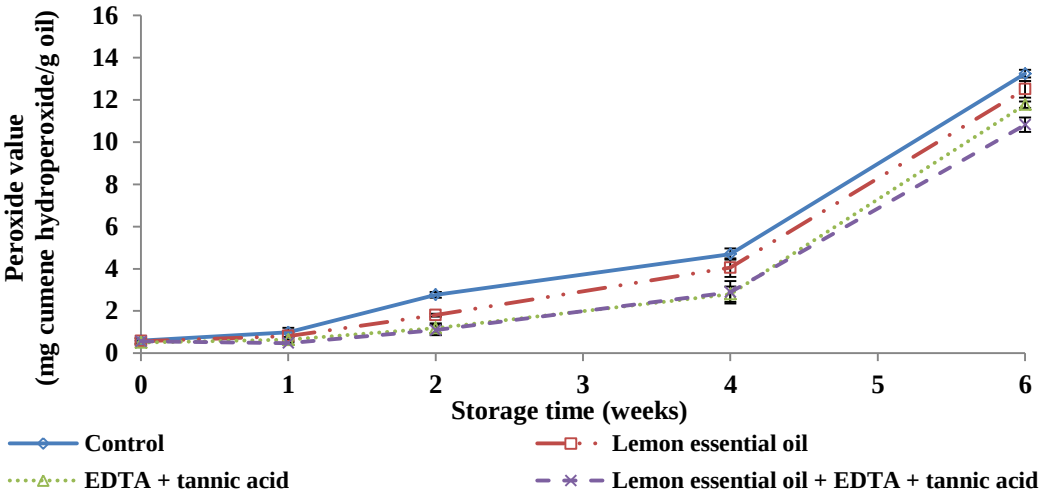

Figure 16 Peroxide values of micro-encapsulated shrimp oil incorporated without and with different antioxidants during the storage of 6 weeks at 30 ºC. Bars represent standard deviations (n=3).

7.5.2.2 Volatile compounds

Volatile compounds in micro-encapsulated shrimp oil without antioxidant and those containing lemon essential oil, EDTA + tannic acid or EDTA + tannic acid + lemon essential oil after 6 weeks of storage are displayed in Table 18. Shrimp oil contained unsaturated fatty acids, in which linoleic acid (C18:2(n-6)) was the dominant fatty acid, followed by oleic acid (C18:1(n-9)) (Takeungwongtrakul *et al.*, 2012). Those fatty acids are prone to oxidation. Volatile compounds in microcapsules (days 0) was also detected. In general, all compounds present in the microcapsule at day 0 were lower in abundance than those found after 6 weeks of storage. Nevertheless, nonanal was also lower in abundance after storage, plausibly due to the volatilization. After storage, the control sample contained several new volatile compounds including 1-penten-3-ol, 2-penten-1-ol, 3-pentanol, 2-hydroxy-3-pentanone, 2-octen-1-al, 3,5-octadien-2-one and 1,4-cyclohexanedione. The highest

amount of lipid oxidation products such as hexanal, 3,5-octadien-2-one, 2-octen-1-al and 2-furanmethanol were found in the control sample after 6 weeks. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acids (Varlet *et al.*, 2006). Aldehydes are the most prominent volatiles produced during lipid oxidation and have been used successfully to follow lipid oxidation in a number of foods (Lee and Decker, 2011) . Amongst all the aldehydic compounds, hexanal was found to be the major aldehydes in control samples, followed by 2-octen-1-al and nanonal, respectively. Hexanal is a typical volatile formed from linoleic acid during both autoxidation and singlet oxygen promoted oxidation (Frankel, 1996). However, 1-octen-3-ol is a volatile generated from linoleic acid oxidation in the presence of singlet oxygen (Lee and Min, 2010).

It was noted that the formation of volatile compounds was reduced when different antioxidants were incorporated in micro-encapsulated shrimp oil. No 2-hydroxy-3-pentanone was found in microcapsule added with antioxidants. Volatile compounds in the sample added with EDTA + tannic acid + lemon essential oil were generally lowest in abundance, compared with those added with other antioxidants. Abundance of volatile compounds in all microcapsules correlated well with the PV values as shown in Figure 16. It was postulated that decomposition of hydroperoxide led to the formation of several volatile compounds (Dlugogorski *et al.*, 2012). The types of volatile compounds detected in micro-encapsulated shrimp oil varied when different antioxidants were incorporated. Different antioxidants with varying modes of action could function in different fashions. The use of EDTA or metal chelator as well as tannic acid and lemon essential oil as radical scavengers effectively prevented oxidation in shrimp oil presented as the core. The combined effect of those antioxidants could be effective in retarding the lipid oxidation, thereby preventing the formation of volatile lipid oxidation compounds related with the rancidity in micro-encapsulated shrimp oil.

Table 18 Volatile compounds in micro-encapsulated shrimp oil in the absence and presence of different antioxidants after storage of 6 weeks at 30 ºC.

Compounds	**Peak area (Abundance) × 10^6**			
	Control	**Lemon essential oil**	**EDTA + tannic acid**	**EDTA + tannic acid + lemon essential oil**
Hexanal	540 (385)*	539	518	390
1-penten-3-ol	354 (ND)	526	420	ND
2-penten-1-ol	135 (ND)	127	103	48
3-pentanol	115 (ND)	ND	67	ND
6-methyl-5-hepten-2-one	ND	117	ND	142
2-hydroxy-3-pentanone	76 (ND)	ND	ND	ND
Nonanal	127 (132)	143	107	104
1-octen-3-ol	88 (37)	90	62	58
2-octen-1-al	234 (ND)	ND	ND	191
1,5-octadien-3-ol	ND	ND	135	ND
3,5-octadien-2-one	306 (ND)	282	ND	ND
Benzaldehyde	ND	ND	152	163
2-nonenal	43 (38)	39	ND	ND
Cyclohexanol, 2-butyl-	ND	ND	37	ND
3,5,5-trimethyl-2-cyclohexenone	ND	125	ND	55
2-furanmethanol	220 (178)	219	198	188
1,4-cyclohexanedione	43 (ND)	39	32	30
2(5H)-furanone	ND	ND	74	ND
4H-pyran-4-one, 3-hydroxy-2-methyl-	177 (125)	137	181	158

ND: non-detectable

* Value in the parenthesis represents the abundance of compound in sample at 0 day.

7.6 Conclusion

Shrimp oil powder made from emulsion containing the mixture of WPC, SC and glucose syrup (1: 1: 2, w/w/w) could improve the EE. The mixture of lemon essential oil, EDTA and with tannic acid effectively inhibited lipid oxidation of shrimp oil microcapsule. Thus, the use of appropriate wall materials in combination the selected antioxidants could yield the shrimp oil microcapsule with high oxidative stability.

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CHAPTER 8

Characteristics and oxidative stability of bread fortified with encapsulated shrimp oil

8.1 Abstract

Characteristics and oxidative stability of bread fortified with micro-encapsulated shrimp oil (MSO) were determined. The addition of MSO could improve the loaf volume of breads. However, chewiness, gumminess and resilience of resulting bread were decreased. Bread crust and crumb showed higher redness and yellowness when MSO was incorporated ($p < 0.05$). Microstructure study revealed that MSO remained intact with bread crumbs. In general, the addition of MSO up to 3% had no adverse effect on bread quality and sensory acceptability. During the storage up to 3 days, no changes in loaf volume and crust color were observed. Oxidation took place in bread fortified with 5% MSO to a higher extent, compared with those with 1 or 3% MSO as indicated by the greater formation of 1-hexanol, nonanal, 1-octen-3-ol and benzeneethanol. Therefore, the bread could be fortified with MSO up to 3%.

8.2 Introduction

Hepatopancreas, a byproduct generated from the manufacturing of hepatopancreas-free whole shrimp, is the excellent source of lipids with high polyunsaturated fatty acids (PUFA) and carotenoids. Nevertheless, oil from hepatopancreas is very susceptible to oxidation, leading to undesirable off-dour (See chapter 2). Rancidity is the major drawback for application of shrimp oil. Encapsulation of oil under the appropriate condition can be a promising means to extend its shelf-life. Encapsulation has appeared as a key technology in delaying or inhibiting oxidation and masking undesirable odor and flavor in the final product. The process involves the conversion of the oil into a free flowing powder, which can be easily handled and used for food fortification. Encapsulation can be defined as a process, in which tiny droplets, namely core, are surrounded by wall materials

(Gallardo *et al.*, 2013). Amongst several encapsulation techniques, spray-drying is the most common micro-encapsulation technology used in food industry due to its low cost, continuous production, ease of industrialization and available equipment (Gharsallaoui *et al.*, 2007).

The world's food market is currently focused on foods that provide nutritive values and health benefits to consumers. Functional foods are rapidly expanding and draw the great attention (Ezhilarasi *et al.*, 2014). Fortification of highly nutritive ingredients such as polyunsaturated fatty acid rich oil, etc. is gaining the interest for food industry. The incorporation of micro-encapsulated oil into foods enables the development of new functional foods with minimal impact on the organoleptic properties of the food products (Ezhilarasi *et al.*, 2014). Wall materials surrounding oil droplets can act as the shield, preventing the oil from oxidation. Borneo *et al.* (2007) fortified micro-encapsulated n-3 fatty acids in cream-filled sandwich cookies without any adverse effect on sensory properties.

Bread has become popular, especially for the new generation (Cleary *et al.*, 2007). The fortification of shrimp oil rich in PUFA and astaxanthin in the encapsulated form could increase the nutritive value of bread. As a consequence, the consumers can intake the active nutrients with the neutraceutical property from the bread. Nevertheless, no information regarding the fortification of micro-encapsulated shrimp oil in bread has been reported.

8.3 Objective

To investigate the effects of micro-encapsulated shrimp oil fortification on the characteristics and sensory property of bread.

8.4 Materials and methods

8.4.1 Chemicals

Ethylenediamine tetraacetic acid (EDTA) was obtained from Merck (Darmstadt, Germany). Tannic acid (99.5% purity) was purchased from Sigma (St. Louis. MO, USA). Essential oils (100% purity) from lemon, plai and basil were obtained from Botanicessence (Bangkok, Thailand). Sodium caseinate was procured

from Vicchi enterprise Co., Ltd. (Bangkok, Thailand). Whey protein concentrate was obtained from I.P.S. International Co., Ltd. (Bangkok, Thailand). Glucose syrup (Dextrose equivalent; 40-43) was purchased from Charoenworrakit Co., Ltd. (Samut Prakan, Thailand). Wheat flour, sugar, salt, shortening, milk powder and yeast were procured from a local market in Hat Yai, Songkhla, Thailand.

8.4.2 Collection and preparation of hepatopancreas from Pacific white shrimp

Hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) with the size of 50-60 shrimp/kg was obtained from the Sea wealth frozen food Co., Ltd., Songkhla province, Thailand during February and March, 2014. Pooled hepatopancreas (3-5 kg) was placed in a polyethylene bag. The bag was imbedded in a polystyrene box containing ice with a sample/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla within approximately 2 h. The sample was stored at -18°C until use, but the storage time was no longer than 1 month. Prior to oil extraction, hepatopancreas was thawed using running water (25°C) and ground in the presence of liquid nitrogen using a blender (Phillips, Guangzhou, China) for 30 sec.

8.4.3 Extraction of oil from hepatopancreas

Oil was extracted from hepatopancreas following the method in chapter 3. The prepared hepatopancreas (20 g) was homogenized with 90 ml of cold solvent mixtures (isopropanal: hexane, 50: 50, v/v) (4°C) at the speed of 9500 rpm using an IKA Labortechnik homogenizer (Selangor, Malaysia) for 2 min at 4ºC. The extract was filtered using a Whatman filter paper No.4 (Whatman International Ltd., Maidstone, England). The residue was extracted with cold solvent mixtures for another two times. The hexane fraction was pooled and repeatedly washed with an equal quantity of 1% NaCl in order to separate the phases and remove traces of polar solvents. Hexane fraction (approximately 135 ml) was then added with 2-5 g anhydrous sodium sulfate, shaken very well, and decanted into a round-bottom flask through a Whatman No.4 filter paper. The solvent was evaporated at 40ºC using an

EYELA rotary evaporator N-1000 (Tokyo Rikakikai, Co. Ltd, Tokyo, Japan) and the residual solvent was removed by nitrogen flushing. The obtained oil was used for micro-encapsulation.

8.4.4 Preparation of shrimp oil-in-water emulsion

Aqueous stock mixed solution of whey protein concentrate, sodium caseinate and glucose syrup at a ratio of 1: 1: 2 (w/w/w) in deionised water was prepared as per the method in chapter 7 Shrimp oil was added into the solution at a core/wall material ratio of 1:4 (v/v). The mixtures were homogenized at a speed of 10,000 rpm for 3 min using a homogenizer (Model T25 basic, IKA Labortechnik, Selangor, Malaysia). The obtained coarse emulsions were then passed through a Microfluidics homogenizer (Model HC-5000, Microfluidizer, Newton, MA, USA) at a pressure level of 4,000 psi for four passes. Emulsions were added without and with lemon essential oil + tannic acid + EDTA. Prior to the incorporation, lemon essential oil (200 ppm) was dissolved in shrimp oil, whereas EDTA (50 ppm) and tannic acid (100 ppm) were dissolved in aqueous stock solution.

8.4.5 Preparation of micro-encapsulated shrimp oil

The prepared emulsions were subjected to drying using a laboratory scale spray-dryer (LabPlant Ltd., LabPlant SD-06A, Huddersfield, UK) equipped with a 0.5 mm diameter nozzle. The emulsion was fed into the main chamber (215 mm diameter × 500 mm long) through a peristaltic pump. Feed flow rate was 8.08 ml/min; drying air flow rate was 4.3 m/s and compressor air pressure was 40.61 psi. Air inlet temperature was 180±2°C. The outlet temperature was controlled at 90±2°C. The obtained powder referred to as micro-encapsulated shrimp oil (MSO) was collected in the amber bottle and capped tightly. MSO contained 18 ± 1.34% shrimp oil and had 1.06 ± 0.05% moisture content. The samples were taken randomly for analyses as follows:

8.4.6 Fortification of MSO in bread

Bread was prepared with the following formulation: wheat flour (500 g), sugar (20 g), salt (8 g), shortening (20 g), milk powder (25 g) and yeast (7 g). Flour and other ingredients were mixed and kneaded uniformly, in which water (300 ml) was added during kneading. Thereafter, MSO was directly added to dough at different levels (0, 1, 3 and 5%, w/w). The dough was kneaded for another 10 min. Bread incorporated with 5% (w/w) wall material powders (without the addition of MSO) was used as the control bread. Dough (150 g) was then subjected to bulk fermentation for approximately 1 h at 30ºC and 75% relative humidity, followed by scaling, intermediate proving, moulding and second proving (for about 1-1.25 h). Finally, baking was carried out at 220ºC for 20 min in baking oven (YXD-20, Guandzhou Xinnanfang electro-thermal equipment Co., Ltd., Guandzhou, China). After baking, bread samples were removed from mold and allowed to cool at room temperature. The bread samples were subjected to analyses.

8.4.7 Characterization of breads fortified with MSO

8.4.7.1 Loaf volume

The volume of bread was determined by sesame displacement method after the loaves were cooled to room temperature for approximately 2 h.

8.4.7.2 Texture profile analysis

Texture profile analysis (TPA) was performed using a TA-XT2 texture analyser (Stable Micro Systems, Godalming, Surrey, UK) with a cylindrical aluminum probe (35 mm diameter). The samples were sliced (each 2.0 cm thickness) and placed on the instrument's base. The tests were performed with two compression cycles. Texture measurements were performed ten times for each sample and mean values were reported. Hardness, springiness, cohesiveness, gumminess, chewiness and resilience were calculated from the force-time-curves generated for each sample (Gökmen *et al.*, 2011).

8.4.7.3. Color measurement

The color of crust and crumb samples were determined using a colorimeter (ColorFlex, Hunter Lab Reston, VA, USA) and reported in the CIE system, including L*, a*, b* and ΔE*, representing lightness, redness/greenness, yellowness/blueness and total difference of color respectively. ΔE* was also calculated using the following equation:

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

where ΔL*, Δa* and Δb* are the differences between the color parameter of the samples and the color parameters of the white standard (L* = 92.85, a* = - 1.20, b* = 0.46).

8.4.7.4 Scanning electron microscopy (SEM)

Bread morphology was evaluated by scanning electron microscopy (SEM). Breads were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed using a scanning electron microscope (Quanta 400, FEI, Eindhoven, Netherlands) at an acceleration voltage of 15 kV with magnification of 2000×.

8.4.7.5 Sensory evaluation

Sensory evaluation was performed by 30 untrained panelists with ages ranging from 20 to 35 years, who were familiar with the consumption of bread. Panelists were asked to evaluate for crust color, crumb color, odor, texture, appearance and overall likeness of bread samples using a nine-point hedonic scale, in which a score of 1 = not like very much, 5 = neither like nor dislike and 9 = like extremely, respectively (Carr *et al*., 1999). Panelists were asked to hand-feel the sample for texture. Bread (2×2×2 cm^3) was served in a closed odorless plastic container at room temperature. The samples were labelled with random three-digit

codes. The order of presentation of the samples was randomized according to "balance order and carry-over effects design" (Heenan *et al*., 2008).

8.4.8 Changes in volatile compounds in bread during storage

Breads were placed in polyethylene bag and sealed. Packaged bread samples with different treatments were stored at room temperature (28-30°C). The samples were taken at day 0 and 3 for analyses. Volatile compounds in breads were analysed by headspace GC–MS using a solid-phase microextraction gas chromatography mass spectrometry (SPME GC-MS) following the method of Gökmen *et al.* (2011) with a slight modification.

8.4.8.1 Extraction of volatile compounds by SPME fibre.

To extract volatile compounds, 1 gram of bread slice was placed in a headspace 20 ml-vial (Agilent Technologies, Palo Alto, CA, USA)). The vial was tightly closed by means of a capper. A carboxen–polydimethylsiloxane solid phase micro-extraction fiber (50/30 µm DVB/Carboxen™/ PDMS StableFlex™) (Supelco, Bellefonte, PA, USA) was used to adsorb the volatile lipid oxidation compounds released from the sample. The fibre was inserted into the vial and equilibrated at 40 °C for 30 min prior to GC–MS analysis.

8.4.8.2 GC–MS analysis

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). The GC oven temperature program was: 35 ºC for 3 min, followed by an increase of 3 ºC/min to 70 ºC, then an increase of 10 ºC/min to 200 ºC, and finally an increase of 15 ºC/min to a final temperature of 250 ºC and holding for 10 min. Helium was employed as a carrier gas with a constant flow of 1 ml/min. The injector was operated in the splitless mode and its temperature was set at 270 ºC. Transfer line

temperature was maintained at 260 ºC. The quadrupole mass spectrometer was operated in the electron ionization (EI) mode and source temperature was set at 250 ºC. Initially, full-scan-mode data was acquired to determine appropriate masses for the later acquisition in scan mode under the following conditions: mass range: 25–500 amu and scan rate: 0.220 s/scan. All analyses were performed with ionization energy of 70 eV, filament emission current at 150 µA, and the electron multiplier voltage at 500 V.

8.4.8.3 Analyses of volatile compounds

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). Quantification limits were calculated to a signal-to-noise (S/N) ratio of 10. Repeatability was evaluated by analysing 3 replicates of each sample. The identified volatile compounds, related to lipid oxidation, including aldehydes, alcohols, ketones, etc., were presented in the term of abundance of each identified compound.

8.4.9 Statistical analysis

All experiments were run in triplicate. All analyses were conducted in five replications. Statistical analysis was performed using one-way analysis of variance (ANOVA). Mean comparison was carried out using Duncan's multiple range test (Steel and Torrie, 1960).

8.5 Results and discussion

8.5.1 Characteristics of bread fortified with MSO

8.5.1.1 Loaf volume

Loaf volume of bread fortified with MSO at different levels is shown in Table 19. Incorporation of MSO (1-5%) resulted in the increase in loaf volume ($p < 0.05$). However, similar loaf volume was obtained, regardless of amount of MSO

added ($p > 0.05$). Loaf volume of breads incorporated with wall material powder (control bread) was not different from that of bread containing MSO at a level of 1% (w/w) ($p > 0.05$). Nevertheless, breads fortified with MSO at the levels of 3 and 5% (w/w) had higher loaf volume than the control bread ($p < 0.05$). Wall material powder, including whey protein concentrate, sodium caseinate and glucose syrup, could increase loaf volume of breads to some degree. Those proteins as well as glucose syrup might strengthen the loaf structure via interaction with wheat gluten, in which the bread matrix could hold gas more efficiently. Whey proteins demonstrated the ability to increase the loaf volume of the breads (Nunes *et al*., 2009). Gluten proteins of wheat flour create unique visco-elastic properties of dough, which allow dough to expand due to the formation of carbon dioxide during fermentation and retain most of this gas inside the dough texture (Wehrle *et al*., 1997). Gökmen *et al.* (2011) and Ezhilarasi *et al*. (2014) reported that increasing amount of micro-encapsulated oil or active compounds decreased loaf volume of bread. Encapsulated substances could decrease the concentration of gluten in the formulation and lower with the retention of gases during the baking process. Based on loaf volume, the addition of MSO up to 5% had no effect on bread quality.

After storage of 3 days at room temperature, in which mold was not detected, no difference in loaf volume was noticeable in comparison with that found at day 0 ($p > 0.05$). Thus, bread structure was not collapsed within 3 days of storage. It was noted that the addition of MSO had no influence on the shelf-life of bread.

8.5.1.2 Textural properties

Textural properties of bread samples containing MSO at various levels are presented in Table 19. The addition of MSO generally had the effects on the texture profile of breads. However, MSO had no effect on hardness ($p > 0.05$), irrespective of amount used. It was noted that, the control bread had higher hardness value than others ($p < 0.05$). The proteins in powder form could be distributed more uniformly and strengthened bread structure more efficiently. Hardness is expressed as the maximum force for the first compression, which relates to the strength of the samples under penetration (Chang *et al*., 2012). For gumminess, the addition of MSO

decreased the value. The decrease was more pronounced as the level of MSO increased ($p < 0.05$). It was found that breads incorporated with 5% of MSO showed the lowest gumminess, compared with others ($p < 0.05$). The addition of MSO or wall material powder had no impact on springiness and cohesiveness ($p > 0.05$). Gumminess is defined as the force required to disintegrate a semi - solid food before it is ready for swallowing. Springiness is a measure of how much the samples structure is broken down by the initial penetration and is calculated as the ratio of the time from the start of the second area up to the second probe reversal over the time between the start of the first area and the first probe reversal. Cohesiveness is a measure of the degree of difficulty in breaking down the internal structure of sample (Chang *et al*., 2012). Chewiness of breads decreased as the amount of MSO in breads increased ($p < 0.05$). Chewiness is related to the time required for masticating a bread piece prior to swallow, and the low chewing value means easy break of the bread in the mouth (Krupa-Kozak *et al*., 2012). Control bread showed similar chewiness to that without MSO ($p > 0.05$). For resilience, breads without MSO (0%) showed the higher value than others ($p < 0.05$). Resilience reflects the redeformation capacity of samples tissue after penetration (Chang *et al*., 2012).

After the storage at room temperature for 3 days, hardness, gumminess and chewiness increased, while the cohesiveness and resilience decreased ($p < 0.05$). Nevertheless, no changes in springiness were observed after the storage ($p > 0.05$). These results indicated that bread staling took place upon storage, mainly due to amylopectin retrogradation (Henna and Norziah, 2011). Therefore, MSO addition had the direct impact on textural property to different degrees, depending on the amount of MSO incorporated.

Table 19 Loaf volume and textural at properties of breads incorporated with MSO at different levels at day 0 and 3 of storage

Storage time (day)	Powders (% w/w)	Loaf volume (ml)	Hardness (g)	Springiness	Cohesiveness (mm)	Gumminess (g.mm)	Chewiness	Resilience
0	**Control**	296.06 ± 9.36bA	1362.22 ± 80.23aB	0.90 ± 0.02aA	0.59 ± 0.02aA	759.25 ± 50.08aB	739.70 ± 38.73aB	0.22 ± 0.01bA
	0 %	269.25 ± 10.84cA	1020.99 ± 54.21bB	0.91 ± 0.03aA	0.61 ± 0.02aA	670.14 ± 57.86abB	712.87 ± 51.31aB	0.24 ± 0.01aA
	1 %	315.22 ± 10.47abA	1022.73 ± 46.70bB	0.91 ± 0.01aA	0.59 ± 0.02aA	622.09 ± 46.49bB	554.13 ± 38.71bB	0.22 ± 0.01bA
	3 %	317.67 ± 9.13aA	938.58 ± 65.57bB	0.90 ± 0.02aA	0.60 ± 0.03aA	610.30 ± 42.41bB	480.51 ± 37.57bcB	0.22 ± 0.01bA
	5 %	317.47 ± 8.86aA	927.85 ± 69.48bB	0.90 ± 0.03aA	0.60 ± 0.01aA	503.91 ± 42.57cA	466.17 ± 47.67cB	0.22 ± 0.02bA
3	**Control**	290.83 ± 8.96bA	2454.22 ± 84.41aA	0.89 ± 0.02aA	0.50 ± 0.02aB	1146.05 ± 54.24aA	1034.57 ± 56.96aA	0.19 ± 0.01aB
	0 %	267.22 ± 11.34cA	2158.52 ± 49.27bA	0.90 ± 0.01aA	0.52 ± 0.02aB	1097.85 ± 39.01aA	979.92 ± 70.76aA	0.19 ± 0.02aB
	1 %	312.17 ± 8.86aA	1532.61 ± 58.13cA	0.90 ± 0.02aA	0.52 ± 0.03aB	838.77 ± 44.37bA	759.42 ± 64.20bA	0.19 ± 0.01aB
	3 %	313.89 ± 10.37aA	1257.53 ± 77.38dA	0.90 ± 0.01aA	0.53 ± 0.01aB	720.33 ± 40.92cA	622.02 ± 59.62cA	0.19 ± 0.01aB
	5 %	313.33 ± 5.77aA	1225.42 ± 34.58dA	0.90 ± 0.01aA	0.52 ± 0.02aB	672.64 ± 54.40cA	617.68 ± 60.45cA	0.17 ± 0.01aB

Control = Added with 5% (w/w) wall material powder without the addition of encapsulated oil.

Data are expressed as mean ± SD (n=3)

Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

8.5.1.3 Color

Color of bread crust was affected by the amount of MSO added as shown in Table 20. The photographs of bread crust are shown in Figure 17A. Bread crust had the decrease in L*-value, but the increases in a*-, b*- and ΔE*- values as the level of MSO increased ($p < 0.05$). Amongst all samples, that added with 5% MSO showed the lowest L*-value but highest a*-, b*- and ΔE*- values ($p < 0.05$). It was found that bread incorporated with wall material powder (control) had the lower L*- value than others, except for that added with 5% MSO. The color of bread crust is mostly attributed to non-enzymatic chemical reactions such as Maillard and caramelization reaction that produce colored compounds (formation of the golden yellow color) during bread baking (Gökmen *et al*., 2011). Proteins in wall material powder could serve as the reactant, especially for browning reaction, especially at crust region. Gökmen *et al*. (2011) reported that the particles in the crust region were partially destroyed due to more severe thermal conditions during baking.

For bread fortified with MSO, the increases in redness (a*- value) were more likely due to the orange/red color of MSO. Shrimp oil contained a high amount of astaxanthin (Takeungwongtrakul et al., 2014). As a result, the bread crust turned to be more orange in color, when MSO was added, especially at higher levels. When comparing L*-, a*-, b*- and ΔE*- values of all bread crust, all bread samples had no change in color after 3 days of storage ($p > 0.05$). The result suggested that the pigments in MSO were stable after 3 days of storage as evidenced by the unchanged color of bread crust. This also confirmed that wall material could protect the oxidation of astaxanthin effectively during the storage.

The color of bread crumb was determined (Table 20). The levels of MSO incorporated in breads were coincidental with the color. The decrease in L*-value and increases in a*-, b*- and ΔE*- values of breads crumb were found as the level of MSO increased ($p < 0.05$). For color of bread crumb, crumb does not undergo Maillard reaction, but is affected by the ingredients in the formula (Conforti and Davis, 2006). Oils from shrimp hepatopancreas were reddish orange in color due to the presence of astaxanthin (Takeungwongtrakul et al., 2014). Additionally, surface oil and oil released to the surface of MSO during bread making could also contribute

to color of bread crumb. When MSO at a level of 5% was incorporated, bread crumb had the lowest L*- value but highest a*-, b*- and ΔE*- values than others ($p < 0.05$). For control bread (with only wall material powder), a*- and b*- values of crumb were not different from those of breads without MSO ($p > 0.05$). Wall material powder was visually white in color without red or yellow color. After 3 days of storage, the control breads had no change in L*- value ($p > 0.05$), while other breads had the increase in L*- value ($p < 0.05$). For a*- and b*- values, all bread crumb had no change in a*-, b*- and ΔE*- values ($p > 0.05$). Nevertheless, crumb of bread incorporated with 5% MSO had the increases in a*- and b*- values. It was noted that those with 0 and 1% MSO had the decrease in ΔE*- value after 3 days of storage ($p < 0.05$).

The photographs of bread crumb are shown in Figure 17B. During storage, the oil might be released from the wall to some degree. As a result, oil with high content of astaxanthin could contribute to the increase in a*- and b*- values to some extent. This was obvious for bread fortified with 5% MSO. Therefore, the addition of MSO directly affected the color of both crust and crumb of bread.

8.5.1 4 Microstrcture

SEM microphotographs of all bread crumbs incorporated with the different levels of MSO are shown in Figure 18. In general, MSO was embedded in the crumb of breads, which was constructed by gluten network. These results were consistent with Gökmen *et al*. (2011) who incorporated nano-encapsulated flax seed oil into breads. Powders added to dough remained intact in the bread crumb. For the control bread, the bead of spray dried wall material was observed throughout the crumb (Figure 18A). However, there was no bead in the bread without MSO and wall material powder (Figure 18B). It was clearly illustrated that the number of breads, representing MSO, increased as the level of MSO increased. In general, MSO were located uniformly in the crumb matrix. Those MSO could serve as the source of PUFA and astaxanthin rich shrimp oil.

Table 20 Color of breads incorporated with MSO at different levels at day 0 and 3 of storage.

Storage time	Powders	Crust color				Crumb color			
(day)	(% w/w)	L*	a*	b*	ΔE*	L*	a*	b*	ΔE*
0	Control	56.16 ± 3.89bA	15.20 ± 2.37bA	36.30 ± 1.36abA	54.28 ± 4.32bA	71.08 ± 1.12aA	0.13 ± 0.02dA	11.67 ± 0.89dA	25.17 ± 1.47eA
	0 %	69.13 ± 2.98aA	9.76 ± 1.12cA	32.12 ± 1.07cA	41.91 ± 1.56cdA	67.66 ± 1.22bB	0.12 ± 0.02dA	11.89 ± 0.60dA	28.35 ± 1.19dA
	1 %	67.88 ± 3.46aA	12.28 ± 1.08bA	35.38 ± 1.06bA	45.42 ± 2.65cA	64.53 ± 1.56cB	2.15 ± 0.41cA	13.72 ± 1.42cA	32.08 ± 1.91cA
	3 %	64.95 ± 2.55aA	13.75 ± 1.17bA	35.73 ± 0.43bA	47.77 ± 1.77cA	63.90 ± 2.07cdB	7.19 ± 0.68bA	18.92 ± 0.91bA	35.89 ± 0.76bA
	5 %	47.27 ± 1.99cA	20.08 ± 0.52aA	38.67 ± 1.28aA	63.47 ± 1.56aA	60.77 ± 1.48dB	11.45 ± 0.51aB	22.59 ± 0.61aB	41.46 ± 0.83aA
3	Control	56.68 ± 3.95bA	14.38 ± 2.26bA	36.31 ± 1.42aA	53.69 ± 0.91bA	72.98 ± 1.09aA	0.12 ± 0.01dA	12.63 ± 1.76dA	23.97 ± 1.36dA
	0 %	67.58 ± 2.16aA	9.67 ± 0.99cA	32.69 ± 0.92cA	42.77 ± 1.88dA	71.99 ± 1.33aA	0.11 ± 0.04dA	12.20 ± 0.76dA	24.61 ± 1.12cdB
	1 %	66.72 ± 2.20aA	11.59 ± 2.17bcA	35.72 ± 1.27bA	46.09 ± 1.09cA	70.94 ± 1.69aA	2.27 ± 0.33cA	13.90 ± 1.28cA	26.55 ± 1.19cB
	3 %	64.10 ± 3.29aA	13.19 ± 2.04bA	35.96 ± 1.10bA	48.28 ± 1.37cA	66.17 ± 2.02bA	7.58 ± 0.36bA	20.13 ± 0.86bA	34.80 ± 1.39bA
	5 %	46.36 ± 0.57cA	20.44 ± 0.69aA	38.54 ± 0.97aA	64.32 ± .041aA	65.11 ± 1.54bA	13.34 ± 0.50aA	26.12 ± 0.99aA	40.90 ± 0.69aA

Control = Added with 5% (w/w) wall material powder without the addition of MSO.

Data are expressed as mean ± SD (n=3)

Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

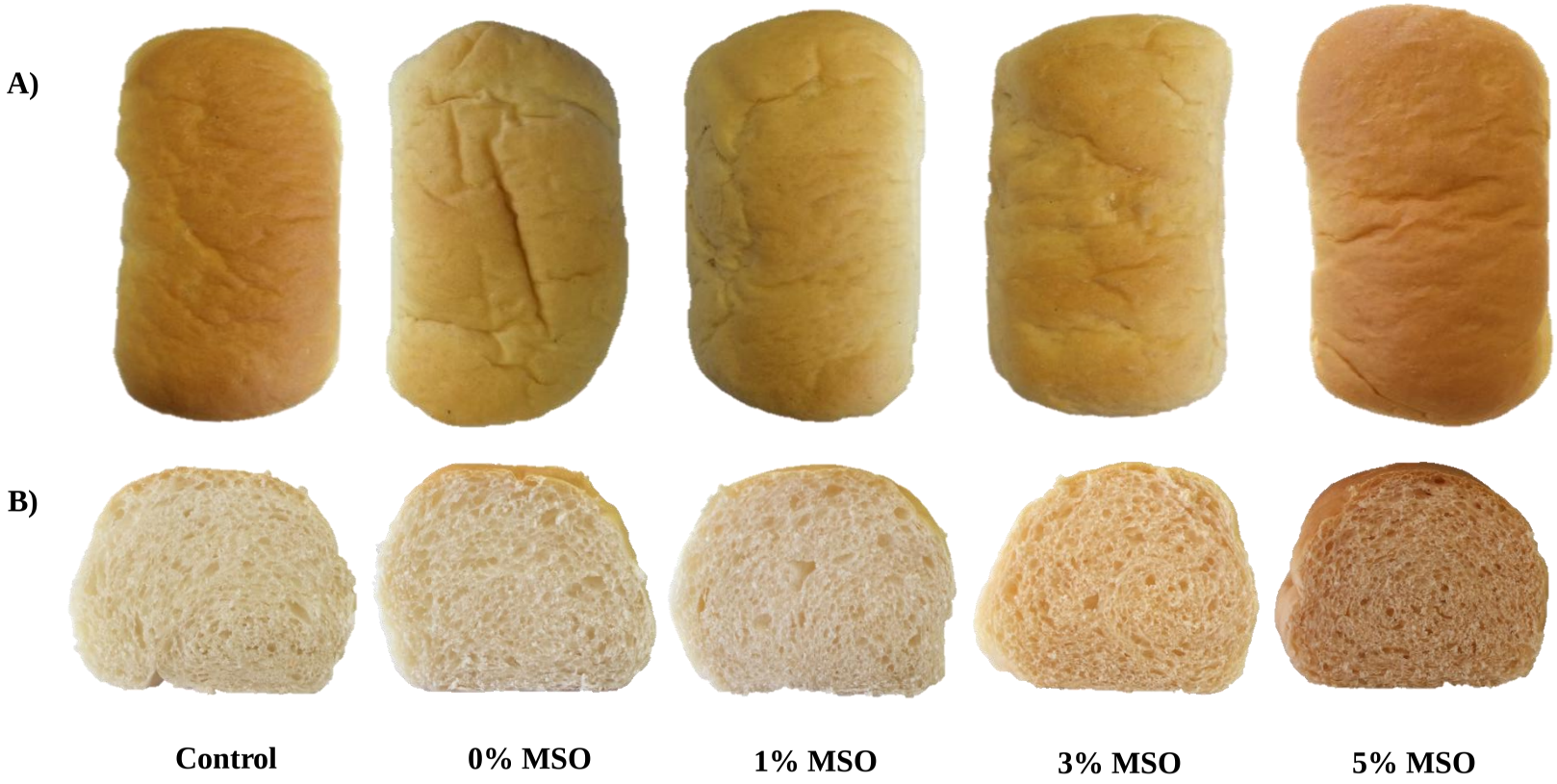
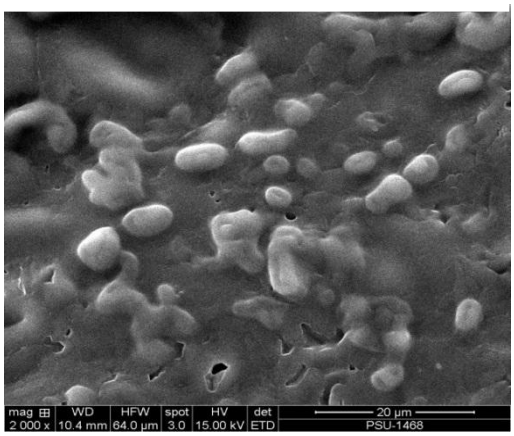
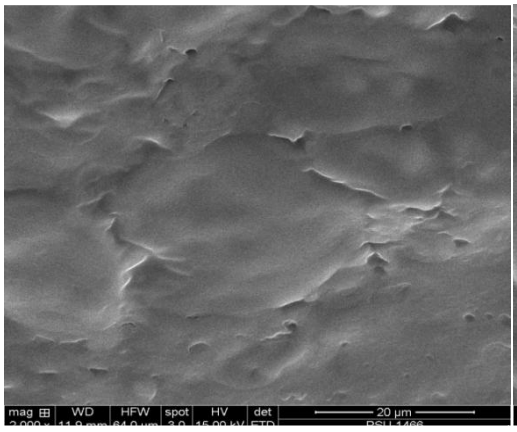


Figure 17 The photographs of breads incorporated with MSO at different levels. A) Bread crust, B) Bread crumb.

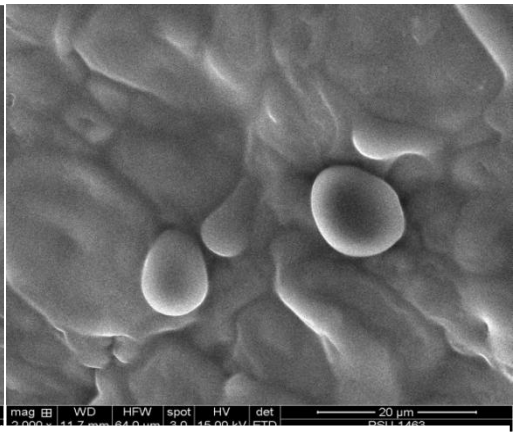
A) Control bread



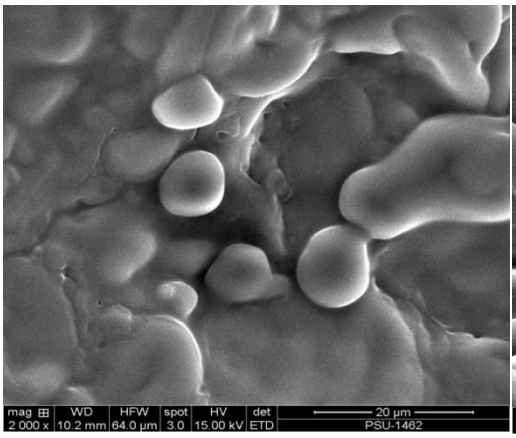
B) Bread with 0% (w/w) MSO



C) Bread with 1% (w/w) MSO



D) Bread with 3% (w/w) MSO



E) Bread with 5% (w/w) MSO

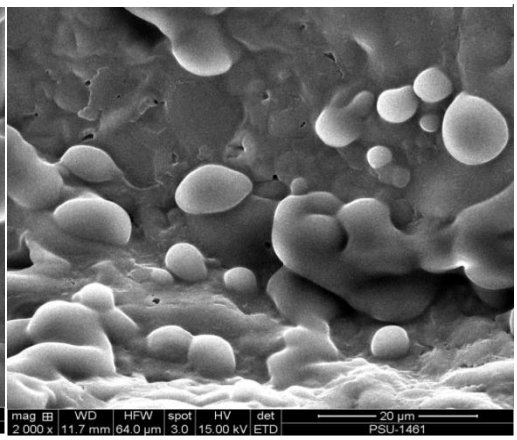


Figure 18 Surface morphology of breads incorporated with MSO at different levels. (Magnification: ×2000).

8.5.1.5 Sensory property

Crust color, crumb color, texture, appearance, odor and overall likeness scores of all bread samples added with different amounts of MSO at day 0 and 3 of storage are shown in Table 21. There were no differences in all attributes amongst all bread samples ($p > 0.05$) at day 0 of storage, except for crumb color and overall likeness scores of breads incorporated with 5% MSO, which had the lower score ($p < 0.05$). The addition of 5% MSO to breads had negative effect on crumb color and overall likeness of breads. This was due to the marked increases in a* and b* values of bread crumb (Table 20). Gökmen *et al*. (2011) reported that the addition of micro-encapsulated n-3 fatty acids could increase functionality of breads. Shrimp oil is one of the important sources of n-3 fatty acids (Takeungwongtrakul *et al*., 2012). Thus, MSO at 3% (w/w) could be added into bread without the negative effect on sensory property of bread.

After 3 days of storage, no differences in all attributes were observed amongst all bread samples ($p > 0.05$). Nevertheless, crumb color, odor and overall likeness of bread added with 5% MSO were lower than others ($p < 0.05$). Wall materials could protect the entrapped core by providing a physical barrier against environmental conditions (Gallardo et al., 2013). Food fortification is good way to induce the general population to consume components, such as n-3 fatty acids, and will add value to food product manufactured by the food industry (Borneo et al., 2007). However, the addition of 5% MSO might result in the increased free oil, especially at the surface of MSO. This led to more free oil, which was susceptible to oxidation. As a consequence, the lower score of odor likeness was found. Therefore, MSO must be incorporated at the appropriate level to avoid the undesirable attributes of bread.

Table 21 Likeness score of breads incorporated with MSO at different levels at day 0 and 3 of storage.

Storage time (day)	Powders (% w/w)	Crust color	Crumb color	Texture	Appearance	Odor	Overall likeness
0	**Control**	7.04 ± 0.78aA	6.90 ± 1.08aA	6.47 ± 1.17aA	7.00 ± 1.04aA	7.13 ± 0.94aA	7.03 ± 1.00aA
	0 %	6.92 ± 0.65aA	6.75 ± 1.16aA	6.70 ± 0.76aA	6.79 ± 0.92aA	7.00 ± 0.94aA	6.90 ± 0.82aA
	1 %	7.04 ± 0.81aA	6.90 ± 0.90aA	7.03 ± 1.27aA	7.07 ± 1.16aA	6.93 ± 0.82aA	6.83 ± 0.95aA
	3 %	7.31 ± 0.89aA	7.10 ± 0.90aA	7.00 ± 0.98aA	6.93 ± 1.00aA	6.97 ± 0.88aA	6.80 ± 0.77aA
	5 %	6.67 ± 0.88aA	5.54 ± 1.12bA	6.97 ± 1.37aA	6.63 ± 1.36aA	6.47 ± 0.95aA	5.63 ± 1.12bA
3	**Control**	6.94 ± 0.89aA	6.90 ± 1.24aA	6.04 ± 0.91aA	7.00 ± 0.89aA	6.52 ± 1.23aA	6.44 ± 0.93aA
	0 %	6.72 ± 0.96aA	6.74 ± 0.96aA	6.38 ± 1.10aA	7.00 ± 1.21aA	6.58 ± 1.31aA	6.36 ± 0.91aA
	1 %	6.83 ± 1.33aA	6.87 ± 1.41aA	6.74 ± 0.90aA	7.06 ± 1.21aA	6.56 ± 1.01aA	6.28 ± 0.96aA
	3 %	7.00 ± 0.80aA	7.00 ± 0.91aA	6.70 ± 0.95aA	6.74 ± 1.21aA	5.96 ± 0.75aA	6.14 ± 1.09aA
	5 %	6.66 ± 1.14aA	5.43 ± 1.07bA	6.60 ± 1.22aA	6.39 ± 1.36aA	5.16 ± 0.94bA	5.36 ± 0.99bA

Control = Added with 5% (w/w) wall material powder without the addition of MSO.

Data are expressed as mean ± SD (n=3)

Lowercase letters in the same column within the same storage time indicate significant difference ($p < 0.05$).

Uppercase letters in the same column within the same sample indicate significant difference ($p < 0.05$).

8.5.2 Volatile compounds

Volatile compounds in bread samples added with MSO at different levels after 3 days of storage are displayed in Table 22. Volatile compounds in bread without MSO (days 0) was also determined. In general, all compounds present in bread without MSO at day 0 were lower in abundance than those found after 3 days of storage. Nevertheless, 3-methyl-1-butanol, 2-pentyl-furan, heptenal and 2-octen-1-al were also lower in abundance after storage, plausibly due to the volatilization or decomposition. After the storage, the bread without MSO contained new volatile compounds including decanal. The highest amount of lipid oxidation products such as 3-methyl-1-butanol, benzeneethanol, benzaldehyde and 2-methyl-1-propanol were found in bread without MSO after 3 days. Several derivatives of aldehyde, ketone and alcohol can be formed by the oxidation of unsaturated fatty acids (Varlet, Knockaert *et al*., 2006). Amongst all the aldehydic compounds, benzaldehyde was found to be the major aldehyde in breads without MSO (0% MSO), followed by nonanal and decanal, respectively. Additionally, volatile ketones (dihydro-5-pentyl-2(3H)-furanone) and volatile alcohols (3-methyl-1-butanol, benzeneethanol, 2-methyl-1-propanol, 1-hexanol, 1-octen-3-ol and 1-octanol) were also found in breads without MSO. Maire et al. (2013) reported that flour appeared relatively rich in alcohols (3-methyl-1-butanol, 1-pentanol, 1-hexanol and 1-octen-3-ol). These compounds were also reported by Hansen and Hansen (1994) in flour with different millings, formed by either lipid oxidation or microorganism metabolism. 1-Octen-3-ol is a volatile generated from linoleic acid oxidation in the presence of singlet oxygen (Lee and Min, 2010). This indicated that lipid oxidation took place in breads without MSO. Maire *et al*. (2013) reported that dough preparation seemed to be the crucial step toward lipid oxidation due to enzymes (lipoxygenase and lipase) as well as air inside the dough texture. Additionally, auto-oxidation could occur during baking by high temperatures, which promote an accelerated oxidation of ingredients in breads without MSO.

After storage, the formation of most volatile compounds in breads increased as the amount of MSO increased from none to 5%. Those compounds included 1-hexanol, nonanal, 1-octen-3-ol, 1-octanol, (Z)-3-decen-1-ol and

benzeneethanol. However, 3-methyl- 1-butanol decreased with increasing MSO. This could be due to volatilization. Benzaldehyde of sample added with 1 and 3% MSO showed the lower abundance than that without MSO. Benzaldehyde formed might bind with protein matrix of bread. Heptenal was also found in the sample added with 5% MSO. Higher abundance in nonanal and benzaldehyde was observed in the sample incorporated with 5% MSO, compared with others after 3 days of storage. Volatile compounds in breads added with 5% MSO were generally highest in abundance, compared with those added with others. With higher level of MSO incorporated, more surface oil susceptible to oxidation could be increased. As a result, oxidation took place to a higher extent. Abundance of volatile compounds in all breads correlated well with the sensory property as shown in Table 21, in which breads added with 5% MSO had the lowest score of odor likeness. For breads added with 1% or 3% MSO and control breads, similar amount of volatile compounds was noticeable and no differences in sensory property of bread were observed ($p > 0.05$) (Table 21). Therefore, 3% MSO was the appropriate level to fortify in bread without negative effect on quality and acceptability.

8.6 Conclusion

Fortification of MSO had impact on the bread quality and sensory properties. MSO up to 3% could be incorporated into bread without affecting its sensorial properties.

Table 22 Volatile compounds in breads incorporated with MSO at different levels after storage of 3 days at 30 ºC.

Compounds	**Peak area (Abundance) × 10^6**				
	Control	**0%**	**1%**	**3%**	**5%**
2-methyl-1-propanol	ND	424 (309)	414	388	264
3-methyl- 1-butanol	1350	1383 (1406)	1293	1264	1255
2-pentyl-furan	ND	ND (128)	ND	ND	ND
3-hydroxy-2-butanone	180	ND	ND	ND	168
Heptenal	ND	ND (74)	ND	ND	118
1-hexanol	414	302 (253)	402	426	441
Nonanal	296	207 (122)	303	366	414
2-octen-1-al	ND	ND (33)	ND	ND	ND
1-octen-3-ol	102	89 (63)	133	176	226
Decanal	41	40 (ND)	26	78	83
Benzaldehyde	347	450 (354)	285	274	477
1-octanol	29	35 (33)	48	52	53
2-octen-1-ol	ND	ND	21	17	20
2-furanmethanol	ND	ND	ND	152	220
(E)-6-nonen-1-ol	89	ND	102	119	ND
(Z)- 3-decen-1-ol	58	ND	59	67	135
Benzenemethanol	22	ND	ND	ND	44
Benzeneethanol	795	716 (683)	728	856	1437
3-hydroxy-2-methyl-4H-pyran-4-one	ND	ND	ND	ND	50
Dihydro-5-pentyl-2(3H)-furanone	ND	19 (15)	23	30	20

ND: non-detectable

* Value in the parenthesis represents the abundance of compound in sample at 0 day.

Control: Added with 5% (w/w) wall material powder without the addition of MSO.

8.7 References

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CHAPTER 9
CONCLUSION AND SUGGESTION

9.1 Conclusions

1. Cephalothorax and hepatopancreas of Pacific white shrimp were susceptible to lipid oxidation and hydrolysis during iced storage. Therefore, the lipid oxidation and hydrolysis should be prevented to maintain the quality of lipids.

2. Extraction of carotenoid containing lipids from shrimp hepatopancreas could be done using the mixture of hexane and isopropanol (50: 50, v/v) with hepatopancreas/solvent ratio of 1: 4.5 (w/v) for three times. Astaxanthin in the lipids played a role in prevention of lipids oxidation during storage.

3. Lemon essential oil was a potential antioxidant in shrimp oil and its effectiveness was comparable to α-tocopherol. However, EDTA in combination with tannic acid effectively inhibited lipid oxidation of shrimp oil-in-water emulsion.

4. Shrimp oil microcapsule was made from emulsion containing the mixture of WPC, SC and glucose syrup (1: 1: 2, w/w/w) with core/wall material ratio of 1: 4 and homogenizing pressure level of 4,000 psi. The mixture of lemon essential oil, EDTA and with tannic acid effectively inhibited lipid oxidation of shrimp oil microcapsule.

5. Fortification of shrimp oil microcapsule into bread had the impact on quality and sensory properties. MSO up to 3% could be incorporated into bread without affecting its sensorial properties.

9.2 Suggestions

1. Enhancement of oxidative stability of bulk or emulsion shrimp oil should be further investigated.

2. Improvement of encapsulation efficiency of shrimp oil should be studied.

3. Decomposition kinetic and stability of astaxanthin in shrimp oil should be elucidated.

4. Fortification of MSO into other food products such as sausage, cookie, etc. should be studied to for enhancement of nutritive value.

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Education Attainment

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Scholarship Awards during Enrolment

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List of Publication and Proceedings

Publications

Takeungwongtrakul, S., Benjakul, S. and H-Kittikun, A. 2012. Lipids from cephalothorax and hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*): Compositions and deterioration as affected by iced storage. Food Chem. 134(4): 2066-2074.

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