LIPOXYGENASE-INDUCED LIPID OXIDATION AND VOLATILE COMPOUNDS FORMATION IN FISH AND SURIMI

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มหาวิทยาลัยเทคโนโลยีสุรนารี

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การเกิดออกซิเดชันของไขมันและสารระเหยโดยการเหนี่ยวนำของเอนไซม์ไลพอกซิจีเนสในปลาและซูริมิ

นางสาววิไลวรรณ กาบศรี

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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วิไลวรรณ กาบศรี : การเกิดออกซิเดชันของไขมันและสารระเหยโดยการเหนี่ยวนำของเอนไซม์ไลพอกซิจีเนสในปลาและซูริมิ (LIPOXYGENASE-INDUCED LIPID OXIDATION AND VOLATILE COMPOUNDS FORMATION IN FISH AND SURIMI)
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คำสำคัญ: ไลพอกซิจีเนส/ออกซิเดชันของไขมัน/สารระเหย/เทคนิคเฮดสเปซ โซลิดเฟสไมโครเอกซ์แทรกชัน-แก๊สโครมาโตกราฟ-แมสสเปกโทรเมตรี (HS-SPME-GC/MS)/ปลานิล/ซูริมิ

การเปลี่ยนแปลงการเกิดออกซิเดชันของไขมันและสารระเหยของเนื้อเยื่อส่วนต่าง ๆของปลานิลได้แก่ เนื้อปลา เหงือก และหนัง ในระหว่างเก็บรักษาในน้ำแข็งถูกตรวจสอบ โดยการวิเคราะห์ค่าเปอร์ออกไซด์ (Peroxide value, PV) กิจกรรมของเอนไซม์ไลพอกซิจีเนส (Lipoxygenase, LOX) องค์ประกอบของไขมันและสารระเหย ตรวจพบกิจกรรมของ LOX และค่า PV ในเหงือก หนัง และเนื้อปลา ตลอดทั้ง 9 วันของการเก็บรักษาและมีปริมาณเพิ่มขึ้นเมื่อระยะเวลาการเก็บรักษาเพิ่มขึ้น กิจกรรมของ LOX มีค่าสูงสุดในเหงือกปลาในขณะที่ค่า PV ตรวจพบสูงสุดในหนังปลา กรดไขมันลดลงระหว่างการเก็บรักษา โดยกรดโอเลอิค (Oleic acid) เป็นกรดไขมันไม่อิ่มตัวมีพันธะคู่เดี่ยว (Monounsaturated atty acid, MUFA) หลัก ขณะที่กรดลิโนเลอิค (Linoleic acid) และกรดโดโคซะเฮกซะอีโนอิก (Docosahexaenoic acid, DHA) เป็นกรดไขมันไม่อิ่มตัวมีพันธะคู่หลายคู่ (Polyunsaturated fatty acid, PUFA) หลักซึ่งพบในทุกส่วนเนื้อเยื่อของปลานิล ตรวจพบสารระเหย 27 ชนิดในเนื้อปลาพบ 45 ชนิดในเหงือกปลาและ 30 ชนิดในหนังปลา เมื่อวิเคราะห์ด้วยเทคนิคเฮดสเปซ โซลิดเฟสไมโครเอกซ์แทรกชัน-แก๊สโครมาโตกราฟ-แมสสเปกโทรเมตรี (Headspace solid phase microextraction - gas chromatography - mass spectrometry, HS-SPME-GC/MS) จากการวิเคราะห์องค์ประกอบหลัก (Principal component analysis, PCA) พบว่า สารระเหยมีการเปลี่ยนแปลงอย่างชัดเจนเมื่อระยะเวลาการเก็บรักษาเพิ่มโดย 2-บิวทาโนน (2-Butanone) และโนนานาล (Nonanal) ในเนื้อปลา 6-เมธิล-2-เฮพทาโนน (6-Methyl-2-heptanone) และ2-โนนานาล (2-Nonanal) ในเหงือกปลา 1-เฮพทานอล (1-Heptanol) และ1-โนนานอล (1-Nonanol) ในหนังปลามีศักยภาพเป็นตัวบ่งชี้ความสดของปลานิล นอกจากนี้เฮกซานาล(Hexanal) สามารถเป็นสารบ่งชี้วัดระดับการเกิดออกซิเดชันของไขมันในปลานิลส่วนต่าง ๆ

LOX มีส่วนทำให้เกิดกลิ่นรสที่ไม่พึงประสงค์ในปลาและอาหารทะเล เมื่อวิเคราะห์กิจกรรมของ LOX ในปลาเขตร้อนที่ใช้ในการผลิตซูริมิ ได้แก่ ปลาทรายแดง ปลาปากคม และปลาหนวดฤษี โดยวิเคราะห์ในส่วนต่าง ๆได้แก่ เนื้อปลา เหงือก และ หนัง พบว่า กิจกรรมของเอนไซม์มีค่าสูงสุดใน

เหงือกของปลาปากคม (376.56 ยูนิตต่อมิลลิกรัมของโปรตีน) ในขณะที่ค่ากิจกรรมของหนังและเนื้อปลาทรายแดงมีค่าสูงสุด (60.67 ยูนิตต่อมิลลิกรัมของโปรตีน) ค่า PV สูงสุดในทุกส่วนของปลาทรายแดง กรดโดโคซะเฮกซะอีโนอิก และกรดอีโคซะเพนตะอีโนอิกเป็นกรดไขมันไม่อิ่มตัวมีพันธะคู่หลายคู่ส่วนใหญ่ที่พบในเนื้อเยื่อส่วนต่าง ๆของปลาเขตร้อน ค่ากิจกรรม LOX ในกระบวนการผลิตซูริมิของปลาปากคมลดลงจาก 157.19 ยูนิตต่อกรัมในวัตถุดิบ เป็น 57.85 ยูนิตต่อกรัมในซูริมิ ซูริมิปลาปากคมมีปริมาณกรดอีโคซะเพนตะอีโนอิกและกรดโดโคซะเฮกซะอีโนอิกสูงแสดงว่า LOX อาจมีส่วนทำให้เกิดออกซิเดชันของไขมันระหว่างกระบวนการผลิตซูริมิและในระหว่างการเก็บแช่เยือกแข็ง LOX จากเหงือกปลาปากคมถูกทำให้บริสุทธิ์ด้วยเซฟาคริวเอส-200 (Sephacryl S-200) และไดเอธิลอะมิโนเอธิลเซฟาโรส (Diethylaminoethyl-Sepharose, DEAE-Sepharose) โดยให้ผลผลิตร้อยละ 3.52 และเพิ่มความบริสุทธิ์ 22.43 เท่า สภาวะเหมาะสมต่อการเร่งกิจกรรมของเอนไซม์คือ 25 องศาเซลเซียส ค่าความเป็นกรด-ด่าง 7.5 เอนไซม์ยังคงแสดงกิจกรรมร้อยละ 80 ที่ 15 องศาเซลเซียส ผลการศึกษาบ่งชี้ว่า เอนไซม์อาจเหนี่ยวนำการเกิดออกซิเดชันของไขมันในระหว่างการเก็บรักษาแบบแช่เย็น กิจกรรมของเอนไซม์ถูกยับยั้งที่ 50 องศาเซลเซียส และมีความเจาะจงต่อสารตั้งต้นกรดอีโคซะเพนตะอีโนอิก เอนไซม์ถูกยับยั้งด้วยกรดเอทิลีนไดเอมีนเตตระอะซิติก (Ethylene diamine tetra-acetic acid, EDTA) เข้มข้น 1 มิลลิโมล และถูกกระตุ้นด้วย 1 มิลลิโมลของเหล็ก (Fe^{2+}) โซเดียม (Na^{+}) และแคลเซียม (Ca^{2+})

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สาขาวิชาเทคโนโลยีอาหาร
ปีการศึกษา 2564

ลายมือชื่อนักศึกษา วิไลวรรณ กาบศรี
ลายมือชื่ออาจารย์ที่ปรึกษา

WILAIWAN KARBSRI : LIPOXYGENASE-INDUCED LIPID OXIDATION AND VOLATILE COMPOUNDS FORMATION IN FISH AND SURIMI. THESIS ADVISOR : ASSOC. PROF. JIRAWAT YONGSAWADIGUL, Ph.D., 97 PP.

Keyword: Lipoxygenase/Lipid oxidation/Volatile compounds/Solid phase microextraction coupled with gas chromatography and mass spectrometry/Tilapia/Surimi

Changes in the lipid oxidation and volatile compounds of various tissues of tilapia (*Oreochromis niloticus*) including muscle, gill, and skin during ice storage were investigated by evaluating peroxide values (PV), lipoxygenase activity (LOX), fatty acid composition, and volatile substances. LOX activity and PV were detected in gill, skin, and muscle throughout 9 days of storage, which increased with extended storage time. The highest LOX was found in gill, whereas the highest PV was found in skin. Fatty acid composition of all tissues decreased during storage. Oleic acid was predominant monounsaturated fatty acid (MUFA), whereas linoleic acid (LA) and docosahexaenoic acid (DHA) were the main polyunsaturated fatty acid (PUFA) in all tissues. Twenty-seven volatile compounds were identified in muscle, while 45 compounds in gill and 30 compounds in the skin based on headspace solid phase microextraction coupled with gas chromatography and mass spectrometry (HS-SPME-GC/MS). Principal component analysis showed gradual changes in the volatile composition with increasing storage time. 2-Butanone and nonanal in muscle, 6-methyl-2-haptanone and 2-nonenal in gill, and 1-haptanol, and 1-nonanol in skin showed the potential to be used as freshness indicators. In addition, hexanal was a potential marker for measuring the degree of lipid oxidation in all tissues.

LOX potentially contributes to oxidative off-flavor in fish and seafood. LOX activities of tropical fish used for surimi production, including threadfin bream, lizardfish, and goatfish were detected in gill, skin, and muscle. The highest LOX activity was found in the gill of lizardfish (376.56 U/mg protein), whereas the highest LOX in skin and muscle was found in threadfin bream of 60.67 and 137.04 U/mg protein, respectively. Threadfin bream showed the highest content of PV. DHA and EPA are the main PUFA in all tissues of tropical fish. LOX activity of lizardfish surimi processing

decreased from 157.19 U/g in raw material to 57.85 U/g in surimi. Lizardfish surimi contained a high amount of DHA and EPA, indicating that LOX could partly contribute to lipid oxidation during surimi production and frozen storage. Lipoxygenase from lizardfish gill was purified by two successive chromatographic steps of Sephacryl S-200 and DEAE Sepharose, resulting in 3.52% yield and a 22.43-fold increase in purity. Optimum activity was found at 25 ° C and pH 7.5 with pH stability at 6.0 - 8.5. There was still 80% of enzyme activity remaining at 15 ° C, suggesting that the enzyme might contribute to lipid oxidation during refrigerated storage of lizardfish. The enzyme was thermally inactivated at 50 ° C. The most preferred substrate was 2.5 mM EPA. The enzyme was inhibited by 1 mM ethylenediaminetetraacetic acid (EDTA) and activated by 1 mM Fe^{2+}, Na^{+}, and Ca^{2+}.

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Student's Signature วิไลวรรณ ภาษศรี
Advisor's Signature

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Wilaiwan Karbsri

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LIST OF ABBREVIATIONS

cm	=	Centimeter
DHA	=	Docosahesaenoic acid (22:6n-3)
EPA	=	Eicosapentaenoic acid (20:5n-3)
FAME	=	Fatty acid methyl esters
g	=	Relative centrifugal fields
GC	=	Gas chromatography
GC-MS	=	Gas chromatography-mass- spectrometry
h	=	Hour
HPLC	=	High performance chromatography
kDa	=	kilo dalton
LOX	=	Lipoxygenase
M	=	Molar
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
MUFA	=	Monounsaturated fatty acids
PCA	=	Principle component analysis
pH	=	Potential of hydrogen ion
PUFA	=	Polyunsaturated fatty acids
PV	=	Peroxide value (primary oxidation product)
sec	=	Second

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LIST OF ABBREVIATIONS (Continued)

SFA	=	Saturated fatty acids
SPME	=	Solid phase microextraction
Std.	=	Standard
U	=	Unit activity
μg	=	Microgram
ω-3	=	Omega 3 fatty acid
ω-6	=	Omega 6 fatty acid
° C	=	Degree Celsius
%	=	Percent

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CHAPTER I
INTRODUCTION

1.1 Introduction

Thailand is a leader in the world's tropical surimi production. Most of Thailand's surimi comes from threadfin bream (*Nemipterus spp*), lizardfish (*Saurida spp*), and goatfish (*Mullidae spp*) (Guenneugues and Lanelli, 2014). Currently, demand for surimi products throughout the world is increasing but traditional resources are limited. Surimi can be produced from freshwater fish like tilapia (*Oreochromis niloticus*), providing good color and gel-forming ability (Rohani, Indon and Yunus, 1995). Tilapia is a prolific species that is easy to cultivate, grows quickly and has a high yield. It has become an important species in terms of help boosting the local aquaculture industry.

Storage of fish in ice is an important post-harvest operation. Biochemical changes of fish at postharvest is species-specific and depends on metabolites in fish tissue, microbial contamination, and post-harvest handlings (Pacheco-Aguilar, Lugo-Sánchez and Robles-Burgueño, 2000). Biochemical changes of tropical fish species at the post-harvest are not well studied as compared to cold water species. Especially, changes in quality of flavor/off-flavor related to biochemical changes have never been systematically investigated despite that fishy note is one of negative attributes of tropical surimi perceived by international market.

Lipoxygenase (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12), is a dioxygenase that oxygenates polyunsaturated fatty acids (PUFA) containing a cis, cis-1,4 - pentadiene structure (–CH=CH–CH_2–CH=CH–), converting them into conjugated unsaturated fatty acid hydroperoxides (ROOH) (Grechkin, 1998). The hydroperoxy fatty acids turn into alcohols, aldehydes, ketones, and hydrocarbons, all of which contribute to an unpleasant taste and odor (Josephson et al, 1984). Marine, and freshwater species are rich in omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic (DHA) eicosapentaenoic acids (EPA), linoleic acid, and linolenic acid

(Strobel, Jahreis, and Kuhnt, 2012). Fish lipids are more prone to oxidation than lipids found in other foods because of their high degree of unsaturation and low antioxidant content. Lipoxygenase-induced oxidation of tropical fish species has not been characterized. It would be important to realize changes of LOX activity from various parts of fish tissues, namely gill, skin, and muscle at post-harvest. A better process control can be designed and implemented for minimizing lipid oxidation and fishy note formation based on this fundamental information.

Previous studies on volatile compounds presume that lipoxygenase (LOX) is a major contributor to the generation of free radicals that cause lipid peroxidation in fish tissue (German, Chen, and Kinsella, 1985). LOX is classified, based on different position of hydroperoxide groups on a fatty acid chain after attacking the 1, 4-cis, cis-pentadiene group. LOX oxidizes at the C13 position of arachidonic acid (AA, 20:4) resulting in hydroperoxide at C15 position, would be called a 15-LOX (Brash, 1999). 1-Octen-3-one, 1-octen-3-ol, 1, (Z)-1,5-octadien-3-ol, and 1, (Z)-1,5-octadien-3-one occurred by action of 12-LOX on EPA, whereas hexanal and (Z)-2-hexenal could occur from **ω**-6 fatty acids and **ω**-3 fatty acids (Cadwallader, 2000). Site specificity of LOXs is a crucial factor in the formation of aroma compounds in fish. Differences in LOX isoenzymes between species are responsible for the distinctive aroma profiles of each species. LOX isozymes have been reported in tissues of various fish, including 12-LOX in trout skin and 15-LOX in the gill of teleost fishes (Hsieh, German, and Kinsella, 1988; German and Creveling, 1990). 5-LOX, 12-LOX, and 15-LOX are present in the gray mullet (Hsu and Pan, 1996). In addition, LOX has a different preference for PUFA, which means that it makes different volatile compounds. Information about LOX specificity of tropical fish used for surimi production is limited thus far.

Typically, surimi has bland taste and less fishy note than its respective mince. However, tropical surimi is known to have strong fishy odor and sometime is considered as off-odor. This would be due to poor post-harvest handling of raw material. Although washing can remove odorous compounds, the extent of strong off-note is much higher than cold water species surimi (An, Qian, Alcazar Magana, Xiong, and Qian, 2020). Hexanal, heptanal, and 1-octen-3-ol are the major volatile compounds of grass carp surimi (Liu et al., 2021). The cause of an off-odor in tropical

surimi has not well understood. Washing is ineffective at removing membrane phospholipids from fish muscle. These phospholipids are PUFA-rich, regularly in contact with muscle heme iron, and highly susceptible to oxidation. (Eymard, Baron, and Jacobsen, 2009). These phospholipids can serve as a substrate of LOX, causing off-flavors. However, it is not known how much LOX residual activity remains after washing. Understanding the role of LOX in flavor characteristic of surimi would lead to better control strategies of surimi processing and storage.

1.2 Research objectives

The objectives of this study were:

1.2.1 To investigate changes of lipoxygenase activity, fatty acid profiles, and volatile compounds of tilapia in various tissues, namely muscle, gill, and skin during ice storage.

1.2.2 To partially purify and characterize lipoxygenase from lizardfish gill (LG-LOX)

1.2.3 To determine the effect of surimi washing process on LOX activity and fatty acid composition.

1.3 Research hypotheses

Ice storage of tilapia would affect LOX activity and extent of lipid oxidation. Volatile compound profiles of muscle, gill, and skin of tilapia are different during ice storage. Principal component analysis could be applied to correlate between volatile compounds and extent of lipid oxidation. Purified LOX from lizardfish gill could be obtained its properties can be characterized. Washing process of lizardfish (LZ) surimi production might reduce LOX activity and fatty acid composition. Residual LOX activity remained after washing would contribute to oxidative off-flavor of surimi.

1.4 Scope of the study

LOX activity, fatty acid profiles, and volatile compounds of tilapia in various tissues, namely muscle, gill, and skin during ice storage were evaluated. Tilapia were stored in ice for 0, 3, 6, and 9 days. Lipid oxidation products were measured by peroxide value. Fatty acid profiles and volatile compounds of various tissues were evaluated.

LOX activity and fatty acid contents of raw threadfin bream, goatfish and lizardfish in various tissues, namely gill, skin, and muscle were evaluated. The effect of industrial production on LOX activity and fatty acid composition was evaluated. Change of surimi LOX activity during LZ surimi processing, namely mince fish, the first washed, second washed, and third washed mince, mince from refiner, screw press, and surimi were followed. LOX of fishes exhibiting the highest activity was selected for purification and characterization. pH optimum and stability, temperature optimum and stability and substrate affinity of purified LOX were evaluated. LOX inhibitors were also tested with various chemicals.

1.5 References

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CHAPTER II
LITERATURE REVIEWS

2.1 Lipoxygenase

Lipoxygenase (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12) is a family of iron-containing enzymes which catalyse the dioxygenation of polyunsaturated fatty acids (PUFA) containing a cis, cis-1,4- pentadiene structure such as linoleic, linolenic, arachidonic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) to yield hydroperoxides (Gardner, 1991). In 1928, LOX in soybean discovered by Bohn and Haas (Bohn and Haas, 1928 cited by Christopher, Pistorius, and Axelrod, 1970). LOX is found in plants, mammals and microorganisms, and is abundant in legumes. LOX in animal tissues has been reported in various aquatic species, showed in Table 2.1. These contain high level of polyunsaturated fatty acids. Skin tissues of 31 species of fish were found to contain pro-oxidant activity, some of which may be attributable to LOX (Mohri, Tokuori, Endo, and Fujimoto, 1999).

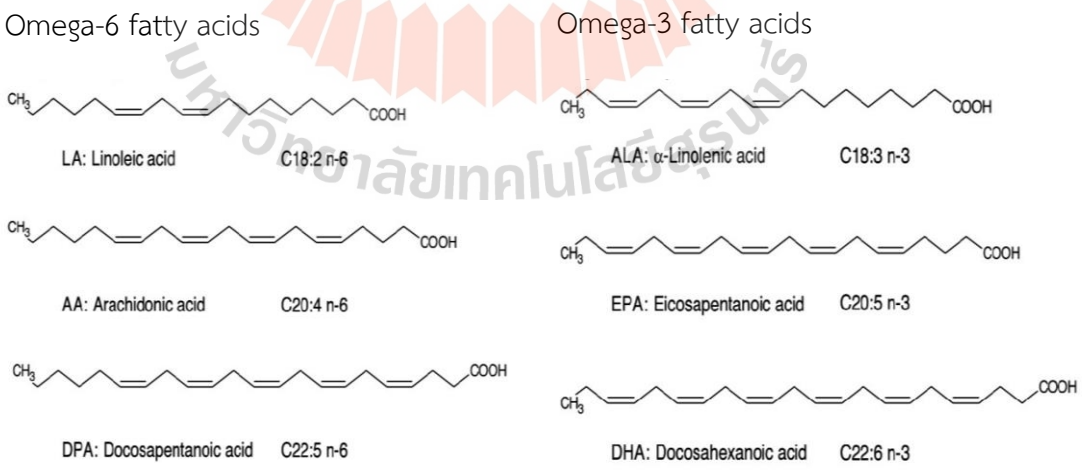

Figure 2.1 Lipoxygenase substrates, omega-6 and omega-3 fatty acids (Kashiwagi, and Huang, 2012).

Table 2.1 Lipoxygenase in various aquatic species

Common name	Scientific name	Part of fish	References
Mackerel	*Scomber scombrus*	Muscle	(BANERJEE, KHOKHAR, and APENTEN, 2002)
White Amur bream	*Parabramis pekinensis*	Muscle	(Ren, et al., 2017)
Rainbow trout	*Oncorhynchus mykiss*	Skin	(German, Chen, and Kinsella, 1985)
Scallop	*Argopecten irradians*	Muscle	(Xie, Yue, and Fan, 2022)
Sardines	*Sardinops melanosticus*	Skin	(Mohri, Cho, Endo, and Fujimoto, 1992)
Nile tilapia	*Oreochromis niloticus*	Skin	(Sae-Leaw, Benjakul, Gokoglu, and Nalinanon, 2013)
Seabass	*Lates calcarifer*	Skin	(Sae-leaw, and Benjakul, 2014)
Baltic herring	*Clupea harengus membras*	Muscle	(Samson, and Stodolnik, 2001)
Sardine	*Sardina pilchardus*	Muscle	(Tolasa Yılmaz, Çaklı, Şen Yılmaz, Kırlangıç, and Lee, 2018)
Grass carp	*Ctenopharyngodon idellus*	Muscle	(Wang, et al., 2012; Cao, et al., 2019)
Lake herring	*Coregonus artedii*	Skin, Muscle	(Wang, Miller, and Addis,1991)
Sorted herring	Clupea harengus	Muscle	(Wu, Forghani, Abdollahi, and Undeland, 2022)
Gray mullet	*Mugil cephalus*	Gill	(Hsu, and Pan, 1996)
Trout	*Salmo gairdneri*	Gill	(German, and Creveling, 1990)
Rockfish	*Sebastes flavidus*	Gill	(German, and Creveling, 1990)
Silver carp	*Hypophthalmichthys molitrix*	Muscle	(Qiu, Xia, and Jiang, 2013; Fu, Xu, and Wang, 2009)
Carp	*Cyprinus carpio*	Gill	(Iijima, Chosa, Hada, and Kayama,2000)

Table 2.1 Lipoxygenase in various aquatic species**.** (Continues)

Common name	Scientific name	Part of fish	References
Rabbit fish	*Siganus fuscescens*	Viscera	(Jiarpinijnun et al., 2022)
Ayu	*Plecoglossus altivelis*	Gill, Skin	(Zhang, Hirano, Suzuki, and Shirai, 1992)
Snakehead	*Channa argus*	Muscle	(Sun et al., 2022)

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2.1.1 Structure

LOXs have a two-domain structure of a smaller -barrel domain (N-terminal domain) and a larger α-helical catalytic domain (C-terminal domain) containing a single atom of non-heme iron essential for activity (Newcomer, and Brash, 2015). In three-dimensional structure, the catalytic domain is primarily an α-helix with a single atom of non-heme iron near its center. The center of the domain consists of two long helices, which donate four iron-binding histidine groups. The fifth group that coordinates the non-heme catalytic iron is the carboxyl group of the C-terminal isoleucine (Figure 2.2). The enzymes from soybean contain 838-865 amino acids, with molecular mass of 94–104 kDa, while 550-600 amino acids, with molecular mass of 70-80 kDa in animal (Brash, 1999). German and Creveling (1990) discovered LOX activity in the gill tissue of teleost fish with molecular mass of 70 kDa. Saeed and Howell (2001) found LOXs with two prominent molecular mass of 119 and 125 kDa in Atlantic mackerel muscle.

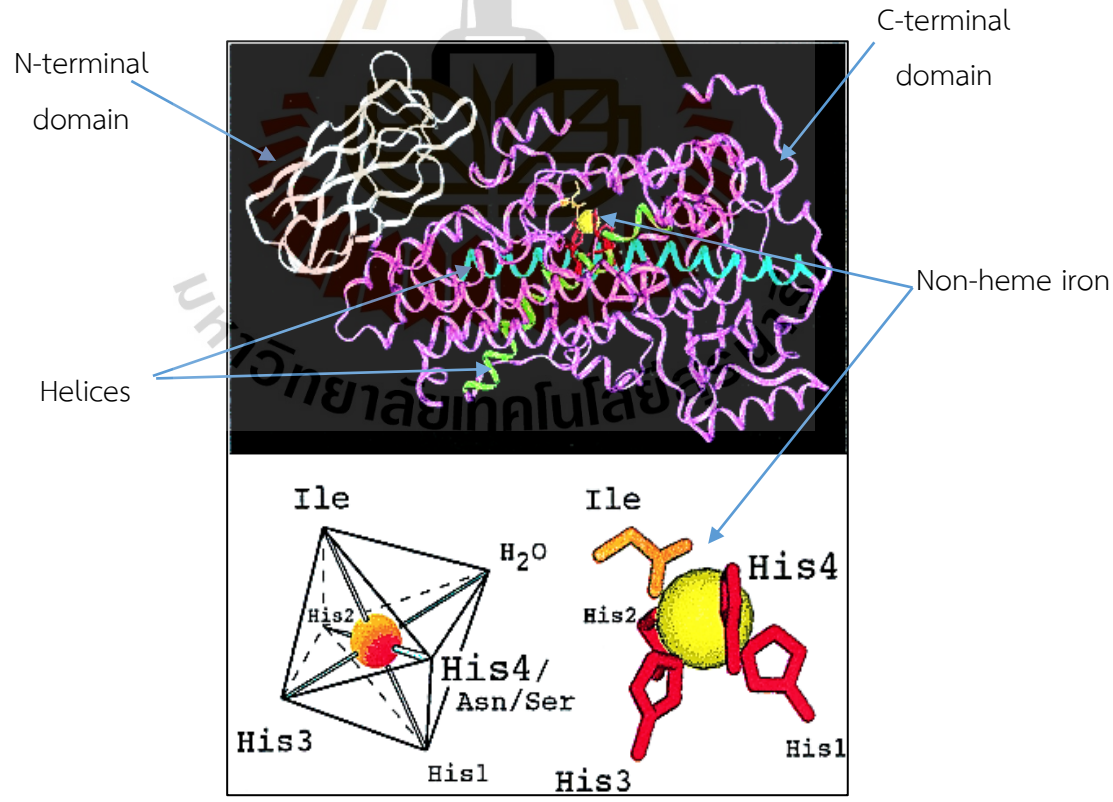

Figure 2.2 Lipoxygenase proteins structure (Brash, 1999)

LOXs are classified, based on different chain lengths of the most prevalent substrates for plants (linoleate) and animals (arachidonate) are used to categorize LOXs. After attacking the 1, 4-cis, cis-pentadiene group, LOX is able to place hydroperoxide groups in various positions along a fatty acid chain. In Figure 2.3, the positional specific of LOX-catalyzed reaction is shown with the main products. In animal tissues, enzymes with specificities for four different positions, including 5-LOX, 8-LOX, 12-LOX, and 15-LOX. For example, LOX oxidizes at the C13 position of arachidonic acid (C20:4), counting from the carboxyl end of the chain, resulting in hydroperoxide at C15 position, would be called a 15-LOX. The products from reaction are hydroperoxy-eicosatetraenes (HPETEs) (Ivanov et al., 2010).

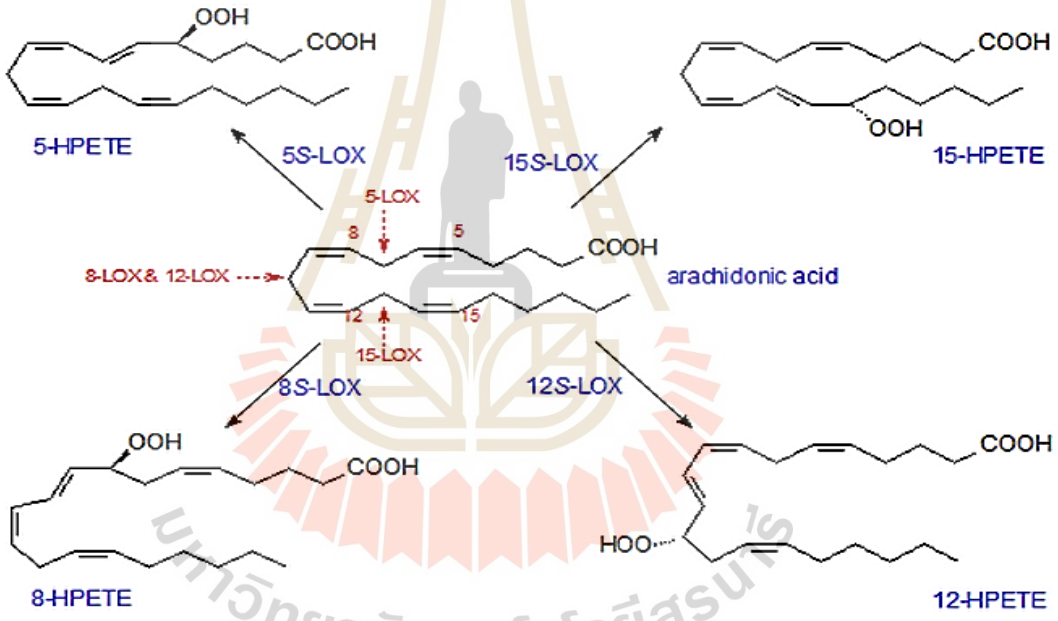

Figure 2.3 Positional specific of LOX-catalyzed reaction with the main products (Brash, 1999)

Hsieh and Kinsella (1988) suggested that LOX activity was present in the gill and skin tissues of several species of fish, and trout exhibited activity of 12-LOXs. German and Creveling (1990) discovered an arachidonic acid 15- LOX activity in the gill tissue of teleost fishes. Moreover, Cadwallader (2000) found that 12-LOX is widely distributed in different organs/tissues of rainbow trout. Three types of arachidonate LOX (5-LOX, 12-LOX, and 15-LOX) are present in the grey mullet (Hsu, and Pan, 1996). Arachidonic acid 12-LOX activity was found in Atlantic mackerel muscle (Saeed and

Howell, 2001). Arachidonic acid 12-LOX protein plays an important role in the occurrence and development of diseases in human (Zheng, Li, Jin, Huang, Zou, and Duan, 2020). Rabbit reticulocyte 15-LOX oxidizes plasma to produce cholesteryl ester and phosphatidylcholine hydroperoxides (Yamashita, Nakamura, Noguchi, Niki, and Kühn, 1999; Morita, Naito, Yoshikawa, and Niki, 2016)

2.1.2 pH optimum and stability

LOX purified from mackerel muscle is stable over the pH range of 4.0-11.0 with pH optimum at 5.6 (Banerjee et al., 2002). German and Creveling (1990) found that gill LOX exhibited activity from pH 7 to 9 with the optimum pH at 7.5. In sardine's skin, the enzyme had an optimum pH at 7.0 and was stable over the pH range of 6.0-9.0 (Mohri et al., 1992). In mackerel's gill; LOX exhibited the highest reactivity toward eicosapentaenoic acid (EPA) with the optimum pH of 4.5. The enzyme was stable at pH 5.5 (Hong et al., 1994). The 12-LOX is widely distributed in different organs/tissues of grey mullet with different optimum pH (gill at pH 8.0, platelet and ovary at pH 6.5) (Cadwallader, 2000). Meanwhile, LOX from soybean exhibited activity at different pHs, depending on isozymes; optimum of pH 9 for LOX 1. pH optimum of LOX 3 is very broad and is centered around pH 7, while the reported pH optimum of seed LOX 2 is 6.8 (Grayburn et al., 1990). Thiansilakul, Benjakul, and Richards (2011) reported the highest lipid oxidation and off-odor were observed in washed Asian seabass mince at pH 6.0.

2.1.3 Thermal optimum and thermal stability

Hsieh et al. (1988) found that at temperatures above 40 ° C, LOX in trout gills was rapidly inactivated and glutathione improved the stability of gill LOX. glutathione can reduce hydroperoxides to its stable hydroxyl analogues and thus removes excessive hydroperoxides to keep the LOX active (Hsu and Pan, 1996). Banerjee et al. (2002) reported that mackerel muscle LOX was stable at 0-50 ° C but lost activity completely at 70 ° C. A decrease in activity is probably due to irreversible changes in the tertiary structure of the enzyme. Moreover, Harris and Tall (1994) reported that After being incubated at 50 ° C for 10 minutes, LOX from mackerel muscle lost 40% of its activity. In mackerel's gill, LOX showed optimum temperature at 25 ° C for EPA, arachidonic acid, and linoleic acid. When linoleic and arachidonic

acid were used as substrates, the highest thermal stability was observed at 8 ° C. When EPA was used as substrates, the highest thermal stability was observed at 20 ° C, indicating that double bonds of EPA were easily oxidized by LOX of mackerel's gill compared to other fatty acids (Hong et al., 1994). Meanwhile, LOX from soy whey protein was inactivated more than 99.9% at 75 ° C for 3 min (Zhang, and Chang, 2022). LOX activity was high in the silver carp surimi gel at 40 ° C, and rapidly decreased at temperature 90 ° C for 10 min (An et al., 2022). Tolasa Yılmaz, Çaklı, Şen Yılmaz, Kırlangıç, and Lee (2018) found that increasing temperature (from 0 to 10 ° C) increased LOX activity significantly in fresh and frozen sardine fillets and mince.

2.1.4 Substrate specificity

The activity of LOX varies with PUFA substrates. Banerjee et al. (2002) reported that the highest activity of mackerel muscle was observed when linoleic acid was used as a substrate, followed by linolenic acid, docosahexaenoic acid (DHA) and arachidonic acid. Substrate specificities of the enzyme appear to vary with sources of LOX. LOX from trout and mackerel efficiently oxidized n-3 fatty acids such as DHA and eicosapentaenoic acid (EPA) (Hsieh al. 1988; Harris and Tall 1994). LOX from carp gill showed maximal activity toward arachidonic acid (Iijima et al., 2000), while sardine skin LOX preferred linoleic acid (Mohri et al., 1990). Gill LOX of rainbow trout exhibited reactivity toward arachidonic, eicosapentaenoic, and docosahexaenoic acids, but low reactivity toward linoleic acid (German and Creveling, 1990). LOX has a different preference for PUFA, which means that it makes different volatile compounds. 1-Octen-3-ol, 1-octen-3-one, 1, (Z)-1,5-octadien-3-ol, and 1, (Z)-1,5-octadien-3-one occurred by action of 12-LOX on EPA, whereas hexanal and (Z)-2-hexenal could occur from n-6 polyunsaturated fatty acids and n-3polyunsaturated fatty acids (Cadwallader, 2000).

2.1.5 Lipoxygenase isozymes

LOX appears to have various isoforms; the products from each isoform are unique. The different LOXs are named related to their positional specificity for the dioxygenation of arachidonic acid: 15-, 12- or 5-LOX, corresponding to an attachment of hydroperoxide to carbon atom 15, 12, or 5 of arachidonic acid, respectively. The 12-hydroxyeicosatetraenoic acid was found to be the major product of LOX (Fu et al., 2009). The 12-LOX is the main LOX in various fish species including trout, sliver carp,

and Atlantic mackerel (Fu et al., 2009; German and John, 1985; Saeed and Howell, 2001). Moreover, 15-LOX is dominant in chicken muscle (Grossman et al., 1988) and trout (German and Richard, 1990). Three form of LOX including, 5-, 12-, and 15-LOX were found in gray mullet gill (Hsiu and Pan, 1996).

2.1.6 Inhibition of lipoxygenase oxidation in fish

The most widely used fish LOX inhibitors are nordihydroguaiaretic acid (NDGA), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), esculetin, ascorbic acid and α-tocopherol. (Grun and Barbeau, 1995; Harris and Tall, 1994; Hsieh, et al., 1988; Mohri, et al., 1999; Saeed and Howell, 2001). NDGA is competitive lipoxygenase inhibitor. Esculetin, known inhibitor of LOX, inhibited the production of 12-hydroxyeicosatetraenoic acid by non-competitive inhibitor as a similar structure to phenolic antioxidants (Saeed and Howell, 2001). Ethylene diamine tetraacetic acid (EDTA) inhibits enzyme-catalyzed oxidation by removing iron. EDTA is more effective in minced meat. Similarly, potassium cyanide (KCN) which acts as a general enzyme inhibitor as well as metal chelator, is also a very efficient heme protein inhibitor. BHT and BHA is an antioxidant which prevents rancidification of food. The conjugated aromatic ring is able to stabilize free radicals by acting as free radical scavengers. In addition, ascorbic acid is a reducing agent for inhibition of enzyme-catalyzed oxidation. Inhibition of crude mackerel muscle LOX by α-tocopherol and ascorbic acid (Saeed and Howell, 2001), inhibition of trout gill LOX by flavonoids (Hsieh et al., 1988) and tilapia and grey mullet gill LOX by green tea extract have been reported (Liu and Pan, 2004). Tea catechins were reported to completely inhibit LOX of all the skin extracts from sardine (Mohri et al., 1999). Carnosine is a dipeptide that reports as a natural antioxidant, which is facilitated by metal ion chelating and peroxyl radical scavenging. Sun et al. (2022) reported the combined application of ultra-high pressure and carnosine on snakehead meat is effective in inhibiting LOX and reducing the fish odor.

2.2 Volatile compounds of fish species

Fish flavor can be divided into three groups: odorless representing in saltwater fish, follow by pyrrolidine an earthy-odor compound representing in freshwater fish and a variety of unsaturated carbonyls and alcohols derived from enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids representing in euryhaline fish.

(Kawai, and Sakaguchi, 1996). Jones et al. (2022) reported different flavor has affect consumer acceptance. Mild ‘fishlike’, ‘marine’, ‘crustacean- like’, ‘seafood’ and ‘sweet’ odors are pleasant. However, "fish-like," "fatty," and "earthy" odors are given as unpleasant. LOX has been reported to contribute to the biogeneration of volatile flavor compounds from the initial oxidation of polyunsaturated fatty acid in fish tissues to produce acyl hydroperoxide (Hsieh, and Kinsella, 1989). The hydroperoxides can be derived from linoleic acid (octadecadienoic acid hydroperoxides, HPODs) acting as substrates for the subsequent enzymatic activities (Zhang et al., 1992; Bisakowski et al., 1997). Hydroperoxide lyase (HPL), reported to cleave the hydroperoxide, producing a volatile compound such as an alcohol or aldehyde (Kermasha, 2002b; Matsui, 2006). Figure 2.4 shows the bioconversion of linoleic acid into alcohol and carboxyl compound by the sequential enzymatic activity of LOX and HPL. LOX is considered as the primary enzyme in the sequential biocatalytic pathway involved in the production of many desirable flavor compounds (Gardner, 1991; Schrader et al., 2004). The first product is the generation of specific HPODs, 9-HPOD and 13-HPODs, considered as flavor precursors. The subsequent catalytic cleavage by the hydroperoxide lyase (HPL), HPODs resulted in their conversion into their corresponding oxoacid and volatile flavor compounds, with distinct flavor characteristics (Figure 2.5).

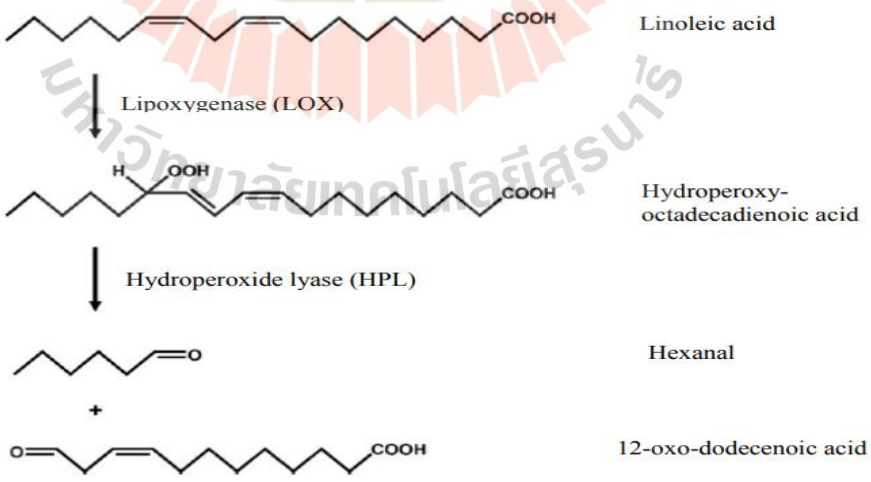

Figure 2.4. Bioconversion of linoleic acid into short-chain alcohols and carbonyl compounds by sequential enzymatic activities of LOX and HPL (Gardner, 1989).

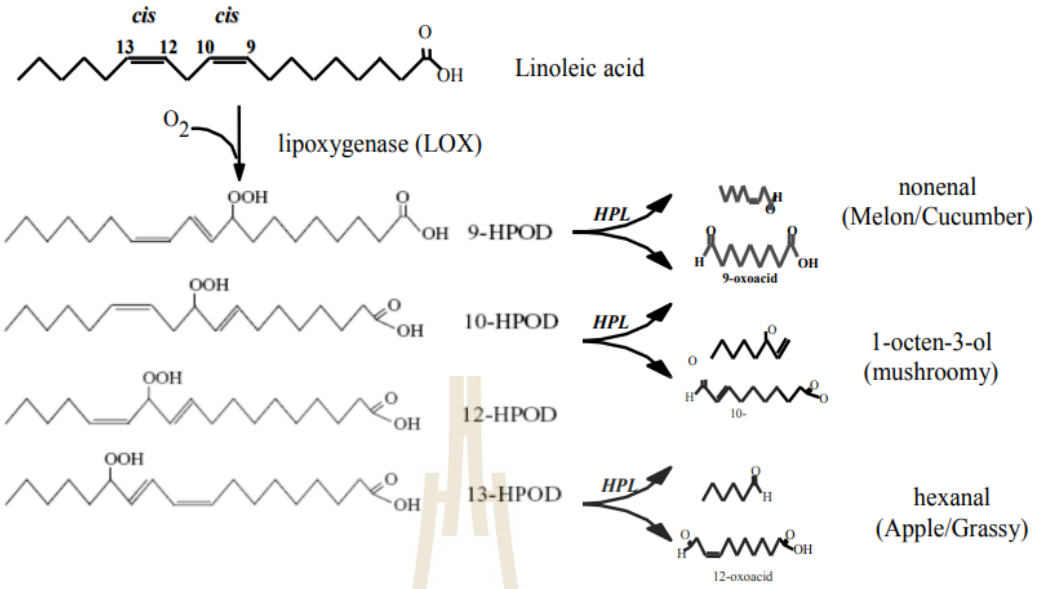

Figure 2.5. The application of lipoxygenase in the production of aroma (Gardner, 1989).

Different species of fish contain different types and activity of LOX, which accounts for different aroma as summarized in Table 2.2. Selli and Cayhan (2009) reported a total of 46 compounds were identified and quantified in gilthead sea bream (*Sparus aurata*) by simultaneous distillation-extraction (SDE) and GC-MS, which aldehydes and alcohols were the most dominant volatiles. (E)-2-Nonenal and decanal were the most powerful contributors to aroma of sea bream. Iglesias et al. (2009) reported that 51compounds were identified and quantified by dynamic headspace with solid phase microextraction (SPME) and gas chromatography–mass spectrometry (GC-MS) in Gilthead sea bream fish. They indicated that 1-octen-3-ol, 1-penten-3-ol, and Z-4-heptenal were markers for the differentiation between fresh and frozen-thawed fish over 226 days. Iglesias and Medina (2008) founded that Atlatic horse mackerel (*Trachurs trachurus*) holding at -20 ° C for 7 months initially contained 15-LOX, while the 12-LOX was barely detected. They also founded a total of 79 compounds identified and quantified by dynamic headspace with SPME and GC-MS. The most dominant volatiles are 1-penten-3-ol and 2, 3-pentanedione (1-octen-3-ol), which are useful indicator of fish rancidity. In silver carp, the major lipoxygenase was 12-LOX. The LOXs caused faster lipid oxidation in the initial phase and it was affiliated with strong fishy odor, which was likely caused by 2, 4-haptadienal (E, E) from the enzymatic oxidation of linoleic acid (Fu et al, 2009). Volatile compounds of fresh

whitefish were identified and quantified by static headspace with SPME and GC-MS. Two distinct families of compounds are cucumber-like note aroma and plant-like aroma. These aromas were identified to be (E)-2-nonenal, (E, Z)-2,6-nonadienal, 6-nonen-1-ol, 1-octen-3-ol, 1-octen-3-one, 1,5-octadien-3-ol, 1,5-octadien-3-one, 2,5-octadien-1-ol (Josephson et al., 1983). Ganeko et al., (2008) postulated that sardine skin contained high levels of polyunsaturated fatty acids and lipoxygenase. Carbonyl compounds may be generated more easily than trimethylamine. They identified 33 volatile compounds of sardine by GC-MS, including 2,3-pentanedione, hexanal, and 1-penten-3-ol. Forty-seven compounds were detected by gas chromatography-olfactometry. Among them, paint-like (1-penten-3-one), caramel-like (2,3-pentanedione), green-like (hexanal), shore-like ((Z)-4-heptenal), citrus note (octanal), mushroom-like (1-octen-3-one), potato-like (methional), insect-like ((E, Z)-2,6-nonadienal), and bloody note (not identified) were strongly detected. It can be concluded that these compounds rather than trimethylamine contributed to fresh sardine flavor. In yellowfin tuna, Edirisinghe et al., (2006) developed a new rapid indicator for determining the quality of fish. Changes in the aroma composition of yellowfin tuna were monitored using SPME GC–MS. The results showed that, hexanal and 2-nonanone were relatively high in fresh fish, whereas 3-methyl-1-butanol and 3-hydroxy-2-butanone increased with storage time.

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Table 2.2 Volatile compounds of fish reported in literatures

Speices	Extraction method	No. of detected compounds	Volatile compounds	Charaterization	Reference
Gilthead seabream (*Spaus aurata*)	Simultaneous distillation-extraction gas chromatography–mass spectrometrometryand SDE/GC-MS	46	Decanal (E)-2-Nonenal	Sea bream aroma	(Selli, and Cayhan, 2009)
Gilthead seabream (*Spaus aurata*)	Solid-phase microextraction gas chromatography–mass spectrometrometry (SPME/GC-MS)	51	1-Penten-3-ol 1-Octen-3-ol Z-4-Heptenal	Fresh and frozen-thawed different markers	(Iglesias et al., 2009)
Silver carp (*Hypophthalmichthys molitrix*)	Solid-phase microextraction gas chromatography–mass spectrometrometryspectrometry–olfactometry (SPME/GC-MS-O)	21	2,4-Heptadienal (E,E) Hexanal Nonanal	Fishy odor Oxidated oil odor	(Fu, Xu, and Wang, 2009)
Atlantic horse mackerel (*Trachurus trachurus*)	Dynamic headspace extraction (DHS) and SPME/GC-MS	79	1-Penten-3-ol 1-Octen-3-ol 2,3-Pentandione	Fishy rancidity markers	(Iglesias, and Medina, 2008)
Sardine (*Sardinops melanostica*)	Gas chromatograph-olfactometry (GC-O) with SPME/GC-MS	33	2,3-Pentanedione Hexanal 1-Penten-3-ol	Sardine aroma	(Ganeko et al., 2008)
Yellowfin tuna (*Thunnus albacares*)	SPME/GC-MS	17	3-methyl-1-butanol 3-hydroxy-2-butanone Hexanal 2-nonanone	Fresh fish markers	(Edirisinghe, Graffham,and Taylor, 2007)

2.3 Analysis of volatile compounds

Sampling methods should be as mild as possible and represent the actual composition of the original sample for analysis of volatiles. One of the methods for volatile compound analysis is headspace analysis. The benefits of using headspace analysis are time saving and simple equipment for sampling. In addition, artefacts caused by heat and contact with solvent and sampling equipment are avoided and volatiles are collected in concentrations presenting their actual vapor pressure in the sample. Temperature has a direct influence on the volatility of the headspace components and sampling conditions and should be chosen so that artefacts induced by temperature will be minimized. Headspace analysis includes static and dynamic headspace. In static headspace, a sample is sealed into a vessel, warmed, and volatile compounds in the sample diffuse into the headspace. Once equilibrium is reached, the sample is withdrawn and injected into the GC column. (Zhao and Barron, 2006). Dynamic headspace is carried out by purge and trap which a flow of carrier gas is passed through the sample vessel to reduces matrix effect and increase the headspace sample size, thus increasing the sensitivity. Volatile compounds are purged from the sample into the headspace above and through a trap, which is rapidly heated and injected into the GC column. Headspace analysis was used to characterize volatile compounds in sea bream (Grigorakis et al., 2013), mackerel (Alasalvar et al., 1997), tuna (Medina et al., 1999), boiled salmon, cod, and trout, and salted-dried white herring (Chung et al., 2007).

Solid phase microextraction (SPME) is another extraction technique developed by Pawliszyn and co-workers in the early 90s that combines sampling and sample preparation in one step (Arthur and Pawliszyn, 1999). SPME can be automated and is easily of use, relatively low cost, and shows high affinity for a large group of compounds. It has become a widely used technique for the isolation of aroma volatiles (Kataoka, 2005). The needle is pushed through a septum and the fiber is contacted to the headspace above the food sample, which is sealed in a suitable container. Volatile compounds are adsorbed onto the fiber and, once equilibrium is reached; the fiber can be removed from the sample vessel and directly desorbed into the split/splitless injector of a gas chromatograph.

Type of fiber separated according to the molecular weights and polarity of the analytes. Non-polar organic volatile compounds with low molecular weight usually require a polydimethylsiloxane (PDMS)-coated fiber. To extract very polar compounds with low molecular weight from polar samples, such as alcohols or amines, polyacrylate (PA)-coated fiber is recommended. Polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fiber is used for aromatic hydrocarbons with small volatile analyses. Larger molecular weight or semivolatile compounds require a fiber with high adsorbtivity and desorptivity including polydimethylsiloxane/ divinylbenzene (PDMS/DVB), Carboxen/ PDMS or DVB/carboxen/PDMS-coated fiber. Several of SPME fibers have been used for volatile of fish. A PDMS fiber was used to examine the effects of storage time on yellowfin tuna (Edirisinghe et al., 2007), while a polyacrylate (PA) fiber was found to be the most suitable for comparing smoked with non- smoked black bream and rainbow trout, included smoked cod and swordfish (Guillen and Erreecalde, 2002, 2006). Song et al. (2002) discovered that aroma of uncooked hepatic tissue was stronger than that of uncooked muscle for carp, flounder, mackerel, and skipjack based on a PDMS/DVB fiber, while DVB/carboxen/PDMS fibers was used to examine sardine freshness (Ganeko et al.,2008). Carboxen/ PDMS fibers was used for analysis of aroma compounds in sea bream, chum salmon, mackerel, sardine, tuna, prawn, and shrimp (Mansur et al., 2003). The HS-SPME has been widely used for the analysis of flavor and freshness in several foods including seafood. These methods have been applied to determine the concentration of aliphatic amines (Zeng et al, 2004), volatiles of yellowfin tuna, sardine (Edirisinghe et al, 2006, Ganeko et al., 2004), differences in volatiles of raw and smoked fish species (Guill and Errecalde, 2002), monitoring the volatile basic nitrogenous species including methylamine, dimethylamine and trymetthylamine for freshness assessment (Chan et al., 2006), differences in volatiles of fresh and fozen-thawed cultured gilthead sea bream fish (Iglesias et al., 2009), the kinetic of lipid oxidation and off-odor formation in silver carp mince (Fu et al., 2009). Iglesias and Medina (2008) applied HS-SPME to determine volatile compounds associated to oxidation of Atlantic house mackerel mince muscle.

2.4 Lipid component in fish muscle

Lipid content and fatty acid profile of fish vary with species and many factors including temperature, salinity, season, size, age, habitat, and others. Freshwater fish in general contain higher proportions of n-6 PUFA (2.42-21.92%) than marine species (0.43–14.2%) (Özogul et al., 2007). Fish can be categorized into fatty, intermediate and lean fish. In fatty fish, fat content is around 8-35 g fat/100g of fish such as herring, sardine and mackerel. In intermediate fatty fish, fat content is 2-8 g fat/100g of fish, such as tilapia and trout. Lean species, such as threadfin bream and Alaska pollack contain 0-2 g fat/100 g of fish (Murray and Burt, 2001). Fish contain high levels of cellular unsaturated lipids which can be converted to hydroperoxides by LOX. Lipids in fish are divided into two main groups, neutral lipids (NL-triacylglycerols, TAG) and phospholipids (PL) (Rehbein and Oehlenschlager, 2009). Phospholipids are made up of four components: fatty acids, a glycerol backbone, a negatively charged phosphate group and a head group of nitrogen-containing alcohol. Triaclyglycerols are less polar hydrophobic, whereas phospholipids are more polar due to phosphate group (Burri et al., 2012).

Phospholipids are divided into three groups: glycerophospholipids, ether glycerolipids and sphingophospholipids. Glycerophospholipids are phospholipids with different polar head 18 groups. For example, phosphatidylcholine (PC) has choline as a head group; while phosphatidylethanolamine (PE) has ethanolamine as a head group, etc. Phosphatidylcholine is a major group in fish, whereas phosphatidylethanolamine (PE) is shown to be the second most abundant. Phospholipids are important component for cell membranes and they function as precursors in structural fat, but the neutral lipids play a role as an energy source (Henderson and Tocher, 1987).

2.5 Surimi

Surimi is a uniquely functional food ingredient. Production of surimi involves heading, gutting and mincing washing, dewatering, and freezing. Good quality surimi is judged by gel-forming ability, water-holding capacity, odorless and creamy white appearance (Park and Morrissey, 2014). Thailand is the leading country for the production of threadfin bream surimi. In addition, surimi can be produced from freshwater fish, like tilapia with good gel-forming ability and white appearance. In addition, odorless is important attribute of surimi (Nopianti, Huda and Ismail, 2010).

2.5.1 Washing process

The washing process is a key step, not only because it removes water-soluble substances, mainly sarcoplasmic proteins, fat and other impurities, like pigments and many metabolic enzymes that reduce the stability of functional proteins during storage. Washing also increases concentration of myofibrillar protein, consequently improving the gel-forming ability. Surimi requires several washing steps to ensure maximum gelling, as well as colorless and odorless surimi (Hall and Ahmad, 1997). However, over-washing lead to substantial loss of fine particles and high moisture content. The number of washing cycles and the volume of water depend on fish species, freshness quality, type of washing solution and the desired quality of the surimi. Normally, two washing cycles using a 3:1 (v/w; water to mince) ratio with a five minutes agitation in each of cycles can be operated to adequate for surimi production (Lee, 1986). Pacheco-Aguilar, Crawford and Lampila (1989) reported that removal of lipids was not efficiently achieved using a single wash with a 3:1 ratio (v/w; water to mince) but resulted in high solids and protein recoveries which are often low in multiple washing cycles. Lin and Park (1996) reported that an effective washing process can now be accomplished with two washing cycles at water to meat ratio of less than 2:1 with a typical wash ratio of 0.9–1.2 parts water in the first wash and 0.7–0.8 in a second wash. Washing process is resulting in substantial loss of gel quality during frozen storage. Hossain et al., (2002) investigated effect of washing solution, washing period and salt concentration on the gel properties of silver carp, they reported that fish mince needed to be washed once with 0.1% NaCl for 10 minutes to obtain a good quality surimi. Manna et al., (2009) reported improving the mince characteristics of marine catfish required to be washed with chilled water (meat: water ratio 1:2) for single time.

2.5.2 Effect of washing on lipid removal

Several research on washing able to decreased lipid concentration around 20-60%. Turan and S**Ö**nmez (2008) reported that the third washing lipid levels decreased approximately 30%. Twice washing reduced lipid level of 64% in the red tilapia and 70% in the Nile tilapia (Biscalchin-Gry ˆschek et al., 2003). In sardine, washing decreased approximately 40% in lipid (Ramirez-Suárez et al., 2000). Acheco-Aguilar et

al. (1989) reported that a single washing at a 3:1 water–flesh ratio and washing at pH 5.0–5.3 were the most effective in removing lipid. Washing three cycles can reduced the fat content by 23% of its initial value in shark meat (Mathew et al., 2002). Pattaravivat et al., (2008) investigated method to decrease the lipid content to less than 1% by washing escolar meat with palmitic sucrose ester (P-1670). After the seconds wash with a 0.25% (w/v) these solutions, the lipid and wax contents decreased to 0.32% to obtain good strength and whiteness of the gel. Barrero and Bello (2000) reported that minced sardine was washed with 0.5% sodium bicarbonate, which removed 57% fat.

Higher phospholipid content in surimi than in mince could be explained by the membrane polar lipids, interacting with proteins and consequently being less easily removed than neutral lipids during washing process (Eymard et al., 2005). Eymard et al., (2005) reported that most of the lipids in horse mackerels mince were removed during washing stage and neutral lipids were lost in higher proportion than polar lipids. Dawson et al., (1990) found that washing removed more neutral lipids than phospholipids from mechanically separated chicken meat. Rhee et al., (1998) reported that washing with high pH (8.2) tap water for 4 times can reduced total fat content, particularly neutral lipids.

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CHAPTER III

CHANGES OF VOLATILE COMPOUNDS FROM LIPID OXIDATION OF NILE TILAPIA DURING ICE STORAGE

3.1 Abstract

Changes in the lipid oxidation and the generation of volatile compounds of various tissues of Nile tilapia, including muscle, gill, skin during ice storage was investigated. Lipoxygenase (LOX) activity and peroxide value were detected in gill, skin, and muscle throughout 9 days of storage and increasing with the extended storage time. The highest LOX was found in gill, whereas the highest PV was found in skin. Individual fatty acid contents decreased during storage. Oleic acid was predominant monounsaturated fatty acid (MUFA), whereas linoleic acid (LA) and docosahexaenoic acid (DHA) were the main polyunsaturated fatty acid (PUFA) in all tissues. Twenty-seven volatile compounds were identified in muscle followed by 45 compounds in gill and 30 compounds in the skin based on headspace solid phase microextraction coupled with gas chromatography and mass spectrometry (HS-SPME-GC/MS). Principal component analysis showed gradual changes in the volatile composition with increasing storage time. 2-Butanone, nonanal, 6-methyl-2-haptanone,1-haptanol, and 1-nonanol can be used as freshness indicators. In addition, 1-penten-3-ol and hexanal were potential markers for measuring degree of lipid oxidation in tilapia stored in ice.

Keywords: Nile tilapia, Lipid oxidation, Lipoxygenase, Solid phase microextraction coupled with gas chromatography and mass spectrometry (HS-SPME-GC/MS), Volatile compound

3.2 Introduction

Tilapia is the world's second most farmed fish, with 6.93 million tons harvested in 2020. (FAO, 2021). It is inexpensive and has white-colored meat, a mild flavor, high protein, and absence of intermuscular bones, making it an acceptable substitute for more expensive choices such as salmon. Fresh fillets of premium-quality tilapia must be processed as soon as possible after harvest, as ice storage reduces the shelf life of the final product (Jimenez-Ruiz et al., 2020). Iced storage is method to maintain the quality of fish during handling and storage. However, fatty fishes are particularly susceptible to lipid degradation which can cause severe problem on postmortem, producing a range of substances having unpleasant taste and smell. Quality deterioration during ice storage of fish is associated with strong fishy note. Hexanal and nonanal are an important compound contributing to fishy odor of seabass skin store in ice for an extended time (Sae-leaw, and Benjakul, 2014). Changes of volatile compounds during ice storage of tilapia have not been well characterized. In this study, changes of volatile compounds during ice storage were monitored. The volatile compounds in tilapia could be useful for determining the freshness and deterioration of the fish during transport/storage.

Fish is a rich in omega-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic (DHA) and eicosapentaenoic acids (EPA) (Strobel, Jahreis, and Kuhnt, 2012). Compared to other food lipids, fish lipids are more susceptible to oxidation due to their high degree of unsaturation and low antioxidant content. Lipid oxidation is associated to the formation of unpleasant odors (Olaoye, 2016). As both lipolysis and lipid oxidation in fish are associated with quality loss, lipid deterioration still occurs readily during storage and limits the shelf life of fish (Pacheco-Aguilar, Lugo-Sanchez, and Robles-Burgue-no, 2000). It is well recognized that various tissues of the fish contain different lipid contents, leading to varied degree of lipid oxidation during ice storage.

Lipoxygenase (LOX) is a dioxygenase that oxygenates polyunsaturated fatty acids (PUFA) containing a 1-cis, 4-cis-pentadiene system converting them into conjugated unsaturated fatty acid hydroperoxides (ROOH) (Grechkin, 1998). The hydroperoxy fatty acids can be further metabolized to produce alcohols, aldehydes,

ketones and hydrocarbons, resulting in off-flavor and off-odors. Previous studies on volatile compounds presumed LOX is a main source of initiating radicals and subsequent lipid peroxidation in fish tissue (German and Kinsella (1985). Mostly LOX has been discovered in muscle, skin, and gill tissue of fish (Tolasa, Çaklı, Şen Yılmaz, Kırlangıç, and Lee 2018; Sae-Leaw, Benjakul, Gokoglu, and Nalinanon 2013; Hsu and Pan 1996). Iglesias et al. (2009) reported that Z-4-heptenal is an important compound contributing to off-note of gilthead sea bream fish, which is produced by the action of LOX on EPA. Moreover, strong fishy odor in silver carp is caused by 2, 4-heptadienal (E, E) resulted from the action of -LOX on linoleic acid (Fu et al, 2009). However, quality changes of tilapia during ice storage as related LOX activity and formation of volatile compounds has not been explored. The aim of this work was to investigate changes of LOX activity tilapia at various tissues, namely gill, skin, and muscle during ice storage.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Bovine serum albumin (BSA), 2-thiobarbituric acid, cumene hydroperoxide, guanidine hydrochloride, trichloroacetic and ferrous chloride were purchased from Fluka (Buchs, Switzerland). Linoleic acid (≥99%), heptadecanoic acid (≥99%), Tween-20, L-glutathione reduced (≥98.0%), phosphatidylcholine, cumene hydroperoxide, cyclohexanol, and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, province, Canada). Other chemicals and reagents were of analytical grade.

3.3.2 Sample preparation

Live tilapia (*Oreochromis niloticus*) with an average weight of 400 – 600 g were purchased from the fish market in Nakhon Ratchasima, Thailand. Fish were placed in ice with a ratio of fish to ice of 1:2 (w/w), packed in polystyrene foam boxes, and immediately transported to the laboratory within 20 min. Upon arrival, the molten ice was drained off and additional ice was added. All polystyrene foam containers were kept in a cold room (4 ℃) throughout the experiment and ice was replaced every 2 days. Temperature was around 0 – 4 °C throughout the storage of 9 days. At 0, 3, 6,

and 9 d of storage, 6 fishes were randomly taken for analyses. Skins were manually removed, gill and flesh were also collected, the skins were washed with cold water, and cut into small pieces using a scissor. Skin, gill, and muscle samples were immediately stored at -70 ° C until use.

3.3.3 Enzyme extraction

Each tissue was homogenized in 0.05 M phosphate buffer (pH 6.5) with 2 mM reduced L-glutathione and 0.04% Tween-20 at ratio of 1:4 (w/v) using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). The homogenate was centrifuged at 15,000 g (Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 20 min at 4 ° C The supernatant was collected and brought to 40% ammonium sulfate, stirred for 30 min and centrifuged at 15,000 g for 20 min. The supernatant was then brought to 70% ammonium sulfate and stirred for 45 min. The enzyme extract was collected from centrifugation at 15,000 g for 30 min. Pellets were collected and dissolved with 0.05 M phosphate buffer (pH 6.5) with 2 mM L-glutathione reduced (≥98.0%) and 0.04% Tween-20, and dialyzed overnight against the same buffer with two changes of the buffer. The extract produced was used and referred to as LOX extract. The temperature was maintained between 0 - 4 ° C at all steps. Protein determination was carried out by the modified Bradford method using bovine serum albumin (BSA) as a standard (Bradford 1976).

3.3.4 Lipoxygenase assay

LOX activity was measured by monitoring conjugated dienes. Linoleic acid was prepared according to the method of Patel, Patel & Thakkar (2015). Linoleic acid (45 mg) was mixed with 90 mg of Tween-20 in 1 ml of DI water. One hundred and fifty microliters of 1 N NaOH was added and the final volume of 20 ml was brought up with 0.05 M phosphate buffer (pH 6.5). The reaction mixture was prepared by adding 1.3 ml 50 mM phosphate buffer, pH 6.5, containing 1 mM glutathione and 0.04% Tween-20 and 100 µl LOX extract. The reaction was initiated by adding 100 µl of substrate. Absorbance at 234 nm of the reaction was recorded every 30 sec. within 3

min. One unit of LOX activity was defined as an increase in absorbance at 234 nm of 0.001 per min.

3.3.5 Peroxide value

Peroxide value (PV) was determined according to the method of Richards and Hultin (2002). Samples (1 g) were homogenized in 11 ml of chloroform/methanol (2:1, v/v). Homogenates were then filtered using a Whatman No. 1 filter paper. Two milliliters of 0.5% NaCl were added to 7 ml of the filtrate. The mixtures were vortexed and then was centrifuged to separate the sample into two phases. To 3 ml of lower phase, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride was added to the mixture. The reaction mixture was kept for 20 min at room temperature prior to reading the absorbance at 500 nm. Blanks were prepared in the same manner, except that deionized water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide/kg sample after blank subtraction.

3.3.6 Total lipid content

Total lipid content of skin, gill and muscle was analyzed according to Bligh & Dyer method (Bligh & Dyer, 1959). Samples (12 g) were homogenized with 60 ml chloroform and methanol solution (2:1, v/v) at 8000 rpm for 2 min at 4 ° C using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). After 30 min, the homogenates were filtered through Whatman No. 1 filter paper into a separating funnel. Thereafter, 24 ml of chloroform, 24 ml of distilled water and 2 ml of 0.58% (v/v) sodium chloride solution were added. The mixture was gentle shaken. After separation, the chloroform phase was drained off into a 125-ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulfate and was shaken very well to remove any traces of water that might be present in the chloroform phase. The solution was filtered through Whatman No. 1 filter paper into a pre-weighed 125-ml Erlenmeyer flask and solvent was removed by flushing with nitrogen. The extracted lipid was stored at -80 ° C for further analysis.

3.3.7 Phospholipid content

Phospholipid content of extracted lipid was evaluated by a colorimetric method as described by Chaijan, Klomklao and Benjakul (2013), based on the formation of a complex between phospholipids and ammonium ferrothiocyanate. One ml of thiocyanate reagent (a mixture of 0.10 M ferric chloride hexahydrate and 0.40 M ammonium thiocyanate) was added to 2 ml of extracted lipid in chloroform solution (0.25 mg/ml). After mixing for 1 min, the lower layer was removed and the absorbance at 488 nm was measured. A standard curve was prepared using phosphatidylcholine (0–0.1 mg/ml) as a standard. Phospholipid content was expressed as mg/100 g lipid.

3.3.8 Fatty acid profile

Fatty acid profiles of extracted lipids from skin, gill and muscle were determined as fatty acid methyl esters (FAMEs) using gas chromatography (GC) according to the AOAC method (AOAC, 2010). Aliquots of the extracted lipids were used to prepare the FAME according to the method of AOAC (2010). In brief, the sample was mixed with 0.5 M methnolic solution (2% sodium hydroxide in methanol), heated at 85 ° C for 30 min, cooled and extracted with 2,2,4-trimethylpentane (Isooctane). Heptadecanoic acid (C17:0) was used as an internal standard. FAMEs were injected, separated and identified on an Agilent/HP 7890 gas chromatograph (Agilent, Palo Alto, CA, USA) with flame ionization detector (FID) equipped with a fused silica capillary column (100 m x 0.25 mm x 0.2 µm film thickness) (Supelco, Bellefonte, PA, USA), using helium as the carrier gas at a flow rate of 1 ml/min. The analytical conditions were: injection port temperature of 250 ° C and detector temperature of 270 ° C. Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid contents were calculated, based on the peak area ratio and expressed as g fatty acid/100 g lipid.

3.3.9 HS-SPME-GC/MS analysis

Each sample (gill, skin, and muscle) was cut into small pieces (0.5x 0.5 cm^2) and 2 g were placed in a 10 ml flat bottom headspace vial fitted with a PTFE/silicone septum and crimp cap (Supleco, Bellefonte, PA, USA) that contained 3

ml of saturated sodium chloride, 30 µl of internal standard (1ppm cyclohexanol) and 10 µl of 7.2% butylated hydroxyanisole (BHA). The samples were analyzed immediately by headspace solid-phase microextraction combined with gas chromatography mass spectrometry (HS-SPME-GC/MS). The sample was pre-incubated at 40 °C for 10 min with agitation. Then, a SPME fiber (50/30 µm DVB/carboxen/ polydimethylsiloxane fiber; Supelco, Bellefonte, PA, USA) was exposed to the vial headspace for 20 min. The fiber was desorbed by splitless injection (injector temperature, 260°C; splitless time, 4min; vent flow, 50 mL/min) into a GC-MS system.

Analyses were carried out using an Agilent GC 7890A series instrument (Agilent Technologies, Inc., Santa Clara, CA, USA). Compounds were separated on a fused silica DB-wax column (60 m x 0.3 mm x 0.25 µm) capillary column. The GC oven temperature program was: 35° C for 3 min, followed by an increase of 3 ° C/min to 70 ° C; then an increase of 10 ° C/min to 200 ° C and finally an increase of 20 ° C/min to a final temperature of 250 ° C, held for 5 min. Helium, with a constant flow of 1.5 ml/min, was used as the carrier gas. Transfer line temperature was maintained at 265 ° C. The identification of the volatile compounds was achieved by comparing their mass spectra with those stored in the National Institute of Standards and Technology (NIST) US Government library. Retention indices of all the volatile compounds were determined by the modified Kovats method reported by Van den Dool and Kratz. MS identification was confirmed by comparing Kovats retention indices (RI) to RI reported in the literatures.

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3.3.10 Statistical analyses

All experiments were conducted in triplicate, and the results were analyzed by one-way analysis of variance (ANOVA). Significant differences in mean values were analyzed by Duncan's multiple range mean comparison test within the 95% confidence interval using All data were analyzed with the SPSS 17.0 statistical package (SPSS Ltd., Working, Surrey, UK). Principal component analysis (PCA) of all measured parameters were performed on means results using the XLSTAT software (Addinsoft, New York, NY, USA).

3.4 Results and discussion

3.4.1 LOX activity and peroxide values of various tissues

LOX activities during ice storage of muscle, gill, and skin of tilapia is demonstrated in Figure 3.1. LOX activities varied with fish tissues. A significant increase in LOX activities was detected in gill, skin, and muscle throughout 9 days of storage ($p < 0.05$). An increase in LOX activities associated with the extended storage time indicated either the increase in extractability of LOX or the activation of LOX in fish tissues. Gill reached its maximum level of LOX activities by 9 days of storage time with a 3.30-fold increase. Moreover, the gill showed the highest LOX activity among other tissues. The increasing rate of muscle LOX activity was slower than that of skin, and reached the maximum level at the end of storage time with a 1.9-fold increase. In the present study, ranking of LOX activity (gill > skin > muscle) agreed with the findings of Wang et al. (2012) who reported that LOX activity of grass carp gradually increased in the muscle and skin less than gill. At 3 days of storage time, muscle and skin showed LOX activity increased significantly, whereas gill showed a significant increase at 6 days of storage time. Membranes and/or subcellular structures of muscle and skin are easily damaged, rendering an increase in LOX activity. Cao et al. (2019) reported LOX activity of grass carp muscle increased significantly during 0–4 days of chilled storage. An increase in LOX activities was also observed in tilapia skin, Baltic herring muscle, and gray mullet gill (Sae-leaw et al., 2013; Samson and Stodolnik, 2001; Hsu and Pan, 1996). Moreover, Medina, Saeed, and Howell (1999) reported that LOX activity of skin tissue of sardine was detected for up to 2 days of chilled storage. Hsieh et al. (1988) reported the high activity of gill LOX at near 0 °C, which is significant in the initiation of fish lipid oxidation under refrigeration conditions. Wu, Forghani, Abdollahi, and Undeland (2022) reported that head showed the highest LOX activity among other fractions of sorted herring and LOX had a significant impact on the lipid oxidation susceptibility. Our results indicate that gill is a main source of LOX in tilapia.

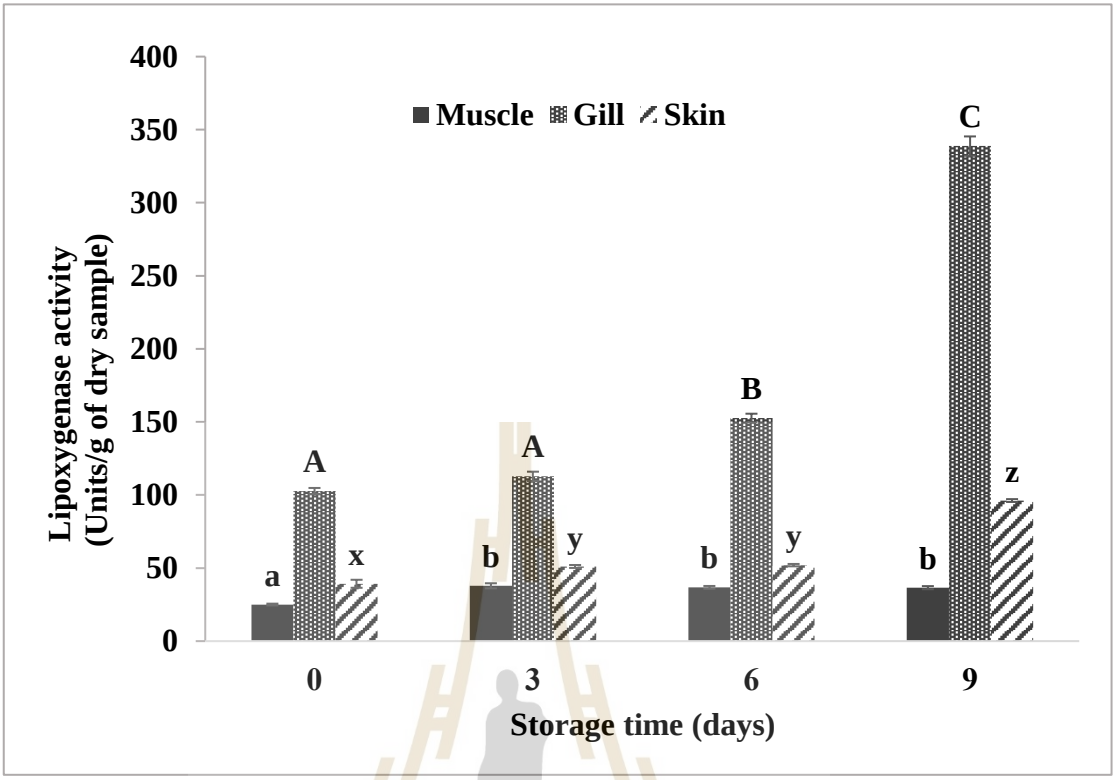

Figure 3.1 Lipoxygenase activity of various tissues of tilapia during ice storage. Means with different superscripts differ significantly ($P < 0.05$) as $^{a-b}$ within the muscle, $^{A-B}$ within gill, and $^{x-z}$ within the skin.

Figure 3.2 showed changes of peroxide values (PV) (mg hydroperoxide/kg of dry sample) of muscle, gill, and skin of tilapia during ice storage. The initial PV of gill, skin and muscle were 2.67, 3.11, and 1.48 mg hydroperoxide/kg of dry sample respectively, which agreed with previously reported values of 1-2 mg hydroperoxide/kg sample in tilapia muscle (Yarnpakdee et al., 2012), and 3-5 mg hydroperoxide/kg sample in sardine muscle (Chaijan et al., 2006). Lipid oxidation occurred during postmortem handling and may also be influenced by muscle type, and age. Fresh fish is known to contain peroxide which is naturally produced and can react with biological molecules. A significant increase in PV was detected in all tissues with storage time ($p<0.05$), suggesting that even at low temperatures, lipid oxidation is always active. Skin showed the highest PV followed by gill and muscle at any storage time. PV values of skin and gill increased by 257% and 247% at 9 days of storage as compared to day 0. Lipid hydroperoxides are formed by either the reaction of singlet oxygen with unsaturated lipids or the lipoxygenase-catalyzed oxidation of PUFA (Nawar, 1996). High

concentrations of polyunsaturated fatty acids (PUFA) were found in all studied tissues, but gill containing the highest PUFA content (Table 1). Yarnpakdee et al. (2012) reported that the increase in PV correlated with the increased amount of non-heme iron, which more likely acted as a pro-oxidant of lipid oxidation. Thus, the degree of oxidation of the muscle, gill, and skin of tilapia kept for an extended time might vary based on a variety of factors, including as the concentration of unsaturated fatty acids, the type and amount of iron, and other pro-oxidants. These lipid oxidation products were detected in the tissues and could have contributed to quality degradation, particularly in fish with an unpleasant flavor. In this study, changes of LOX activity doses not correspond with PV. LOX activity was highest in gill, but PV was highest in skin. It might be that LOX are not the main factor in lipid oxidation. The extent of lipid oxidation can be influenced by various factors, including oxygen consumption, the concentration of prooxidants, pH, temperature, and fatty acid content, which affect skin more than gill tissue.

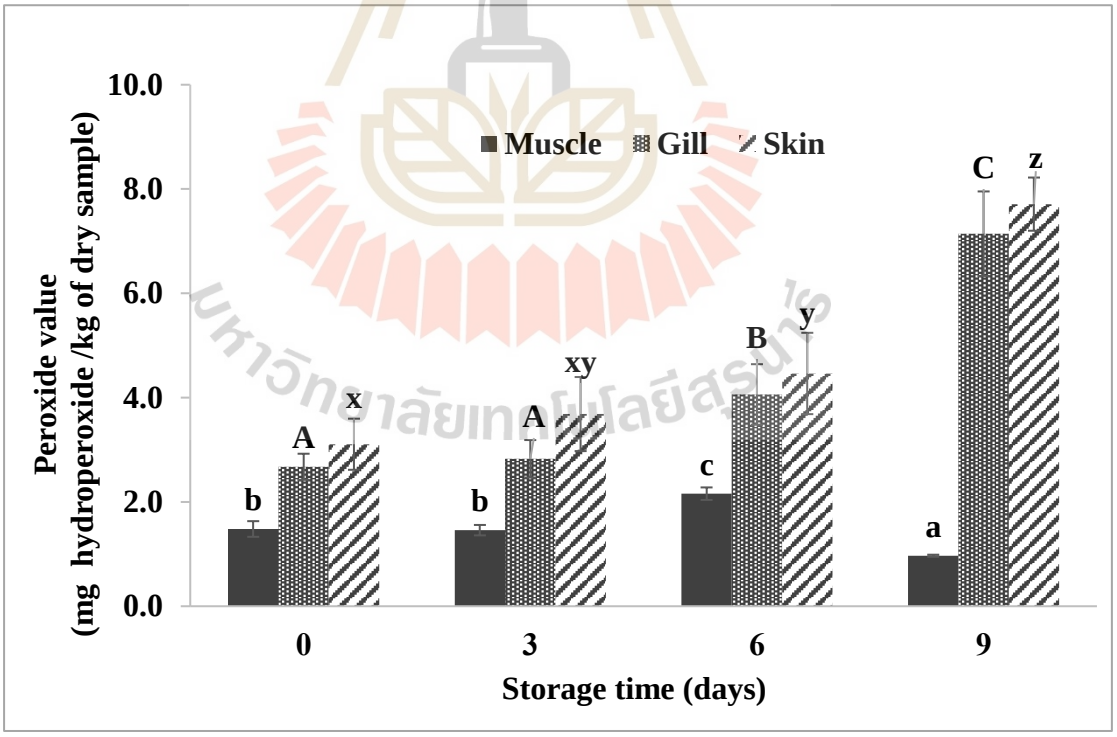

Figure 3.2 Peroxide values of various tissues of tilapia during ice storage. Means with the different superscripts differ significantly ($P < 0.05$) as $^{a-b}$ within the muscle, $^{A-B}$ within gill, and $^{x-z}$ within the skin

3.4.2 Changes of fatty acids at various tissues during ice storage

Changes in the content of various fatty acids in different tissues of tilapia could indicate the extent of lipid oxidation. Changes of fatty acids during ice storage of muscle, gill, and skin and of tilapia are listed in Table 3.1. Gill showed the highest fatty acid contents followed by skin and muscle. A decrease in fatty acid was detected in all tissues at day 9 of storage time ($p<0.05$). Lipid oxidation is likely to be a main reason for the decrease in fatty acids, which coincided with an increase in PV content (Fig. 3.2). Moreover, activation of lipoxygenase is believed to have caused such decreases in fatty acids (Joaquin, Tolasa, Oliveira, Lee, and Lee, 2008). However, Sae-leaw et al. (2013) reported that fatty acid content of tilapia skin increased significantly during 18-day storage. It was possibly due to lipase activity and phospholipase activity. Gill and skin showed a decrease in ∑MUFA and ∑PUFA in the first 3 days, whereas muscle showed a decrease in 6 days ($p<0.05$). Skin showed the highest rate of decrease in ∑MUFA and ∑PUFA among other tissues, coinciding with the highest PV. The decreasing rate of ∑PUFA was higher than ∑MUFA in all tilapia tissues studied, indicating that a high degree of unsaturated lipid were easily oxidized. Lipid oxidation includes autooxidation and enzymatic oxidation. LOX is a significant endogenous enzyme related to free fatty acid oxidation in fish (German, Chen, and Kinsella, 1985). In our study, palmitic acid as the predominant saturated fatty acid (SFA), oleic acid is highest monounsaturated fatty acid (MUFA), whereas linoleic acid (LA) and docosahexaenoic acid (DHA) are the main polyunsaturated fatty acid (PUFA) in all tilapia tissues. Xu et al. (2019) reported that unsaturated fatty acid in silver carp mince, particularly PUFA, significantly decreased during cold storage. Since the whole fish were stored in ice, LOX from intracellular might be released and initiated lipid oxidation, converting DHA and EPA into more polar products (German and Kinsella, 1985). Mori, Cho, Endo, and Fujimoto (1992) found LOX in sardine skin oxidized linoleic acid more efficiently than arachidonic acid and EPA. LOX in mackerel gill exhibited the highest reactivity toward EPA followed by arachidonic acid and linoleic acid (Hong, Shim, and Byun, 1994). LOX in rabbit fish muscle with an arachidonic acid (ARA) substrate specificity contributes to volatile lipid oxidation products that explain the unpleasant smell of rabbit fish (Jiarpinijnun et al., 2022). Wu et al. (2022) reported that LOX had a significant impact

on lipid oxidation susceptibility, although the quantity of lipids or polyunsaturated fatty acids had no significant correlation.

3.4.3 Changes of volatile compounds

Figure 3.3 shows GC-MS chromatograms obtained from the SPME analysis of muscle, gill, and skin from tilapia at day 9 of ice storage. Twenty-seven volatile compounds were identified in muscle followed by 45 compounds in gill and 30 compounds in the skin (represented by relative content %), grouped by chemical families (Table 3.2): alcohols, aldehydes, ketones, furans, acid, ester, hydrocarbons, and aromatic compounds. In our study, the majority of these compounds showed significant statistical difference ($p<0.05$) after ice storage compared to initial values, indicating that ice storage affects the volatile nature of tilapia.

Alcohols were found to be the compounds showing the highest peak areas during ice storage in muscle, gill, and skin. Among them, 1-hexanol showed the highest level in the muscle and skin at the end of storage.1-Hexanol has been reported as a major volatile compound of fish, derived from the degradation products of oleic acid and palmitic acid, which were the major fatty acids in tilapia tissue. Whereas, 1-octen-3-ol and 1-nonanol showed major alcohols in gill. 1-Octen-3-ol is an important contributor to off-flavor in fish due to its low odor score and generated from the oxidation of arachidonic and eicosapentaenoic acids (EPA) by gill lipoxygenase and degradation of linoleic acid hydroperoxide (Hsieh and Kinsella, 1989; Iglesias et al., 2009; and Mu et al., 2017). Jiarpinijnun et al. (2022) reported that 1-octen-3-ol was the main volatile compound associated with the unpleasant smell in rabbit fish generated from the reaction of LOX and arachidonic acid (ARA).

Table 3.1 Fatty acid compositions of muscle, gill, and skin from Nile tilapia during iced storage.

Fatty acids (mg/ g of dry sample)	Muscle				Gill				Skin			
	0 day	3 days	6 days	9 days	0 day	3 days	6 days	9 days	0 day	3 days	6 days	9 days
C10 : 0	ND	ND	ND	ND	ND	ND	ND	0.06 ± 0.01	ND	ND	0.01 ± 0.01	0.01 ± 0.02
C11 : 0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.01 ± 0.02	0.01 ± 0.01
C12 : 0	0.29 ± 0.02	0.26 ± 0.01	0.27 ± 0.02	0.29 ± 0.01	1.04 ± 0.04	0.95 ± 0.04	0.8 ± 0.02	0.84 ± 0.07	0.48 ± 0.07	0.38 ± 0.04	0.34 ± 0.02	0.27 ± 0.02
C13 : 0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C14 : 0	2.45 ± 0.17	2.31 ± 0.13	1.88 ± 0.19	2.25 ± 0.17	9.29 ± 0.49	8.47 ± 0.99	7.72 ± 0.31	6.53 ± 0.49	3.94 ± 0.76	2.73 ± 0.34	2.81 ± 0.13	2.04 ± 0.19
C15 : 0	0.14 ± 0.02	0.21 ± 0.01	0.1 ± 0.02	0.12 ± 0.01	0.55 ± 0.04	0.57 ± 0.04	0.42 ± 0.06	0.46 ± 0.03	0.23 ± 0.05	0.18 ± 0.02	0.16 ± 0.01	0.12 ± 0.01
C16 : 0	21.51 ± 0.68	21.67 ± 0.22	18.17 ± 0.86	20.49 ± 1.4	81.6 ± 1.54	73.44 ± 1.46	63.58 ± 0.84	62.29 ± 2.02	36.09 ± 7.35	23.99 ± 2.61	22.9 ± 0.61	17.78 ± 0.63
C17 : 0	4.87 ± 0.07	4.32 ± 0.14	4.64 ± 0.06	2.98 ± 0.02	16.34 ± 0.31	14.52 ± 0.5	11.9 ± 0.37	17.24 ± 0.35	6.62 ± 0.05	7.09 ± 0.12	6.92 ± 0.1	5.86 ± 0.13
C18 : 0	5.63 ± 0.56	5.17 ± 0.19	4.56 ± 0.21	5.13 ± 0.41	22.6 ± 1.35	18.16 ± 0.88	14.98 ± 0.3	15.72 ± 0.76	9.89 ± 2.18	5.98 ± 0.51	5.55 ± 0.14	4.58 ± 0.24
C20 : 0	ND	ND	ND	ND	ND	ND	ND	0.53 ± 0.01	ND	0.79 ± 0.07	0.81 ± 0.06	0.6 ± 0.04
C21 : 0	0.21 ± 0.05	0.23 ± 0.03	0.18 ± 0.02	0.16 ± 0.03	0.87 ± 0.03	0.76 ± 0.07	0.62 ± 0.12	0.29 ± 0.03	0.34 ± 0.02	0.12 ± 0.02	0.11 ± 0.02	0.09 ± 0.01
C22 : 0	0.09 ± 0.02	0.07 ± 0.01	0.06 ± 0.02	0.07 ± 0.01	0.25 ± 0.04	0.31 ± 0.08	0.27 ± 0.02	0.04 ± 0.07	0.08 ± 0.06	0.78 ± 0.06	0.75 ± 0.03	0.61 ± 0.07
C23 : 0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
C24 : 0	0.65 ± 0.05	ND	ND	0.31 ± 0.15	ND	ND	ND	ND	0.76 ± 0.1	ND	ND	ND
ΣSFA	**35.8 ± 1.58^{c}**	**34.21 ± 0.7^{bc}**	**29.83 ± 1.36^{a}**	**31.76 ± 2.18^{ab}**	**132.52 ± 2.18^{d}**	**117.15 ± 1.75^{c}**	**100.26 ± 1.85^{b}**	**103.93 ± 2.05^{a}**	**58.4 ± 10.62^{b}**	**41.99 ± 3.75^{a}**	**40.34 ± 1.11^{a}**	**31.92 ± 1.32^{a}**
C14 : 1	0.12 ± 0.01	0.13 ± 0.01	0.08 ± 0.02	0.11 ± 0.01	0.41 ± 0.06	0.38 ± 0.04	0.36 ± 0.04	0.34 ± 0.04	0.18 ± 0.02	0.13 ± 0.02	0.15 ± 0.02	0.11 ± 0.01
C15 : 1	ND	ND	ND	ND	ND	ND	ND	0.04 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.03	0.01 ± 0.01
C16 : 1	4 ± 0.15	4.7 ± 0.2	3.71 ± 0.42	3.95 ± 0.4	14.44 ± 0.65	14.17 ± 0.79	12.49 ± 0.12	12.43 ± 0.84	6.57 ± 1.27	4.46 ± 0.54	4.6 ± 0.29	3.47 ± 0.18
C17 : 1	0.14 ± 0.02	0.21 ± 0.04	0.16 ± 0.05	0.13 ± 0.01	0.51 ± 0.08	0.62 ± 0.11	0.46 ± 0.03	ND	0.24 ± 0.03	ND	ND	ND
C18 : 1n9t	0.46 ± 0.06	0.23 ± 0.05	0.19 ± 0.05	0.38 ± 0.06	1.45 ± 0.14	1.11 ± 0.11	0.67 ± 0.06	0.72 ± 0.42	0.68 ± 0.08	0.35 ± 0.04	0.37 ± 0.02	0.16 ± 0.14
C18 : 1n9c	**32.24 ± 0.19**	**31.9 ± 0.53**	**29.53 ± 0.87**	**28.31 ± 2.01**	**113.6 ± 1.36**	**108.52 ± 2.73**	**92.91 ± 1.74**	**88.48 ± 2.14**	**51.32 ± 8.77**	**32.86 ± 2.88**	**31.31 ± 1.31**	**24.83 ± 0.99**
C20 : 1	1.1 ± 0.07	0.99 ± 0.02	0.96 ± 0.07	0.99 ± 0.04	3.67 ± 0.35	4.15 ± 0.2	2.94 ± 0.09	2.41 ± 0.11	1.88 ± 0.34	1.77 ± 0.18	1.57 ± 0.06	1.02 ± 0.31
C22 : 1n9	0.19 ± 0.01	0.11 ± 0.03	0.09 ± 0.04	0.16 ± 0.01	0.67 ± 0.02	0.69 ± 0.14	0.46 ± 0.04	0.67 ± 0.03	0.28 ± 0.05	0.13 ± 0.02	0.12 ± 0.01	0.09 ± 0.01
C24 : 1	ND	0.72 ± 0.06	0.34 ± 0.05	ND	1.96 ± 0.08	1.2 ± 0.16	1.13 ± 0.07	0.21 ± 0.02	0.00 ± 0.00	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
ΣMUFA	**38.23 ± 0.47^{b}**	**38.96 ± 0.91^{b}**	**35.03 ± 1.54^{a}**	**33.98 ± 2.52^{a}**	**136.67 ± 1.85^{d}**	**130.8 ± 2.22^{c}**	**111.39 ± 1.84^{b}**	**105.26 ± 2.42^{a}**	**61.11 ± 10.53^{c}**	**39.74 ± 3.66^{b}**	**38.19 ± 1.71^{ab}**	**29.73 ± 1.63^{a}**
C18 : 2n6t	ND	ND	ND	ND	ND	ND	ND	0.08 ± 0.06	0.00 ± 0.00	0.03 ± 0.03	0.04 ± 0.04	0.02 ± 0.02
C18 : 2n6c	**14.75 ± 0.89**	**14.24 ± 0.17**	**13.23 ± 0.77**	**12.99 ± 0.15**	**48.99 ± 1.32**	**50.31 ± 1.23**	**40.14 ± 0.39**	**39.4 ± 1.73**	**22.8 ± 3.89**	**15.72 ± 1.41**	**13.38 ± 1.5**	**10.99 ± 0.57**
C18 : 3n6	1.38 ± 0.14	1.3 ± 0.09	0.63 ± 0.02	0.79 ± 0.1	4.64 ± 0.41	3.4 ± 0.22	2.26 ± 0.23	2.04 ± 0.1	1.67 ± 0.42	0.21 ± 0.02	0.18 ± 0.01	0.14 ± 0.01
C18 : 3n3	1.5 ± 0.11	1.23 ± 0.04	1.08 ± 0.1	1.33 ± 0.09	5.1 ± 0.28	4.57 ± 0.12	3.92 ± 0.13	3.28 ± 0.2	2.3 ± 0.34	1.33 ± 0.12	1.17 ± 0.05	0.9 ± 0.06
C20 : 2	0.73 ± 0.05	0.64 ± 0.01	0.63 ± 0.05	0.64 ± 0.03	2.43 ± 0.1	2.29 ± 0.15	1.83 ± 0.07	2.03 ± 0.07	1.05 ± 0.18	0.06 ± 0.01	0.06 ± 0.01	0.26 ± 0.26
C20 : 3n6	0.79 ± 0.07	0.77 ± 0.03	0.58 ± 0.06	0.72 ± 0.03	2.43 ± 0.21	2.04 ± 0.15	1.64 ± 0.08	2.2 ± 0.11	1.07 ± 0.19	ND	ND	ND
C20: 3n3	1.03 ± 0.14	1.19 ± 0.05	0.54 ± 0.1	1.01 ± 0.01	3.06 ± 0.1	3.19 ± 0.42	2.35 ± 0.12	0.32 ± 0.01	1.7 ± 0.32	0.29 ± 0.03	0.26 ± 0.02	0.19 ± 0.02
C20 : 4n6	0.11 ± 0.01	0.12 ± 0.02	0.06 ± 0.01	0.11 ± 0.01	0.43 ± 0.13	0.34 ± 0.08	0.3 ± 0.04	2.46 ± 0.22	0.16 ± 0.02	1.06 ± 0.05	1.23 ± 0.02	0.87 ± 0.05
C22 : 2	ND	ND	ND	ND	ND	ND	ND	0.14 ± 0.05	ND	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
C20 : 5n3	0.09 ± 0.02	0.14 ± 0.02	0.09 ± 0.02	0.12 ± 0.04	0.38 ± 0.1	0.38 ± 0.07	0.22 ± 0.04	0.74 ± 0.07	0.19 ± 0.07	0.19 ± 0.02	0.18 ± 0.01	0.16 ± 0.04
C22 : 6n3	**1.58 ± 0.08**	**1.41 ± 0.12**	**0.65 ± 0.22**	**1.33 ± 0.08**	**4.71 ± 0.18**	**4.88 ± 1.24**	**2.11 ± 0.17**	**3.08 ± 0.23**	**3.8 ± 1.95**	**1.25 ± 0.08**	**1.18 ± 0.01**	**0.84 ± 0.05**
ΣPUFA	**21.92 ± 1.48^{b}**	**20.98 ± 0.53^{b}**	**17.46 ± 1.3^{a}**	**19.02 ± 0.51^{a}**	**72.13 ± 1.32^{b}**	**71.37 ± 0.71^{b}**	**54.75 ± 0.51^{a}**	**55.72 ± 2.57^{a}**	**34.7 ± 7.35^{c}**	**20.14 ± 1.74^{b}**	**17.69 ± 1.64^{ab}**	**14.37 ± 1.04^{a}**

*SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

$^{a-d}$ Mean values in the same row with different superscripts differ significantly (P < 0)

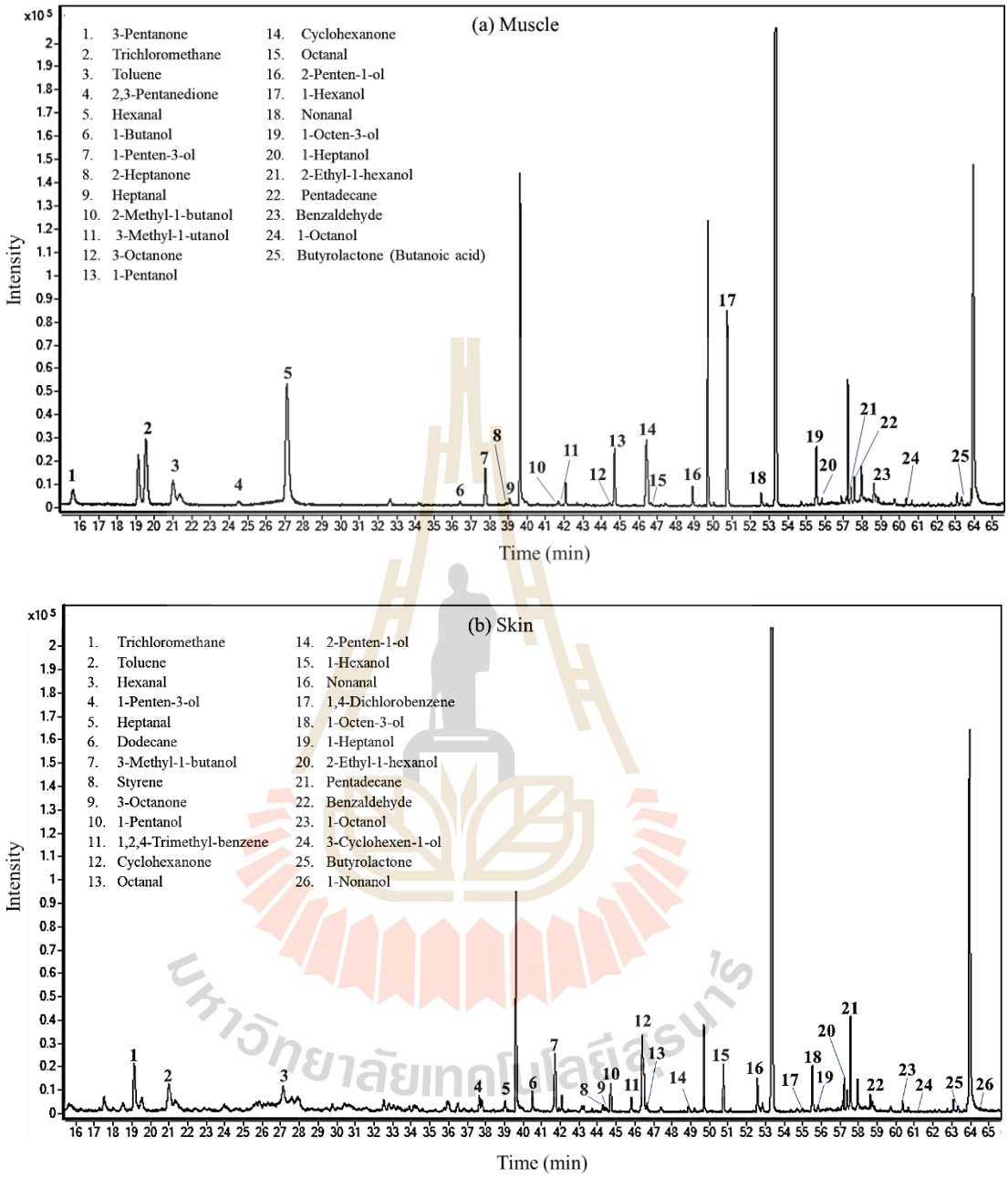

Figure 3.3 Representative GC–MS (full scan) chromatogram of muscle (a), skin (b), and gill (c) from Nile tilapia at day 9 of ice storage.

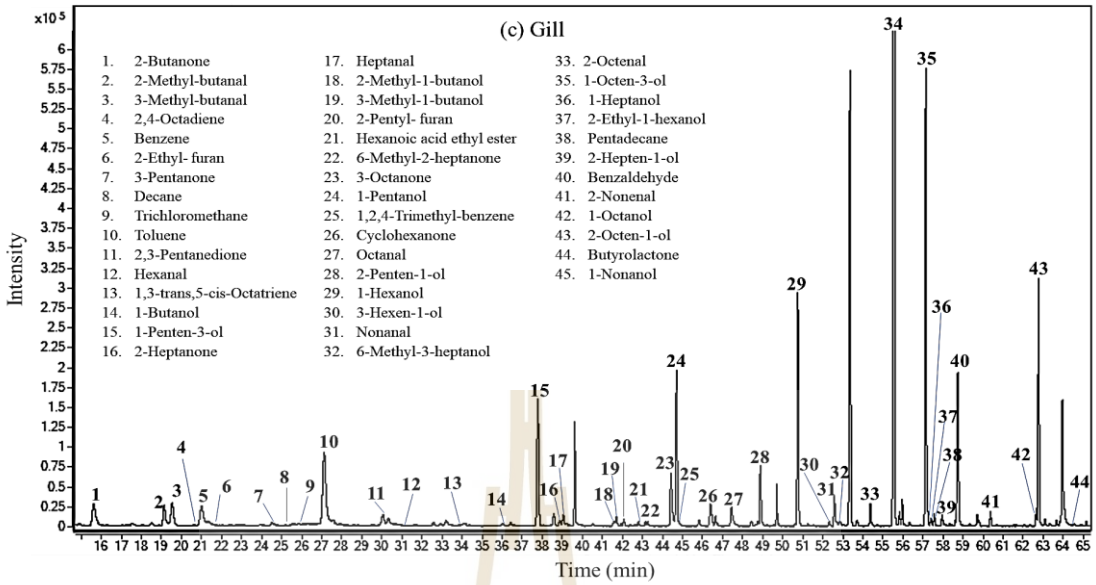

Figure 3.3 Representative GC–MS (full scan) chromatogram of muscle (a), skin (b), and gill (c) from Nile tilapia at day 9 of ice storage. (Continued).

A significant increase in 1-penten-3-ol and 2-penten-1-ol was detected in gill, skin, and muscle at day 9 of storage ($p<0.05$, Table 3.2). An increase of 1-penten-3-ol can be explained by lipoxygenase acting on EPA and hydroperoxide lyases, which is used as a marker of lipid oxidation in chilled Atlantic horse mackerel muscle (Iglesias and Medina, 2008). The increase of 2-penten-1-ol can be explained by oxidized lipid, which was the most potent odorant of raw sardine (Prost et al., 2004). 3-Methy-1-butanol was identified in muscle, gill, and skin of tilapia with the increase of storage time ($p<0.05$). Similar trend has already been observed in previous studies, which caused an unpleasant smell and was derived from oxidative deamination of leucine by spoilage bacteria (Wang, Chen, Zhang, Wang, and Shi, 2019).

Table 3.2 Relative amount of volatile compounds detected by HS-SPME-GC/MS in tilapia during ice storage.

Compound name by class	RIL	RIC	Muscle				Gill				Skin			
			0	3	6	9	0	3	6	9	0	3	6	9
Alcohols (17)														
1-Butanol	1158	1156	0.43 ± 0.06^{ab}	0.33 ±0.07^{a}	0.56 ± 0.03^{c}	0.49 ± 0.09^{bc}	0.10 ± 0.01	0.1 ± 0.01	0.11 ± 0.07	0.13 ± 0.02	ND	ND	ND	ND
1-Penten-3-ol	1166	1169	1.56 ± 0.20^{a}	2.08 ± 0.23^{a}	4.32 ± 0.21^{a}	3.97 ± 0.38^{b}	4.07 ± 0.31^{a}	5.11 ±0.85^{ab}	7.91 ± 0.63^{b}	5.29 ± 1.11^{a}	2.27 ± 0.46^{a}	2.26 ± 0.63^{a}	1.27 ± 0.04^{a}	5.32 ± 0.95^{b}
1-Butanol, 2-methyl-	1205	1211	ND	0.25 ± 0.07^{b}	ND	0.18 ± 0.01^{a}	0.14 ± 0.02	0.13 ± 0.04	0.13 ± 0.03	0.08 ± 0.01	ND	ND	ND	ND
1-Butanol, 3-methyl-	1208	1212	0.15 ± 0.00^{a}	0.11 ± 0.01^{a}	ND	0.32 ± 0.08^{b}	0.05 ± 0.01^{ab}	0.06 ± 0.02^{ab}	0.10 ± 0.01^{b}	0.30 ± 0.01^{a}	0.40 ± 0.05^{a}	0.5 ± 0.07^{a}	0.94 ± 0.11^{a}	7.63 ± 0.77^{b}
1-Pentanol	1259	1257	4.64 ± 0.75	5.17 ± 0.72	3.89 ± 0.49	4.68 ± 0.96	3.71 ± 0.37	3.48 ± 0.22	4.78 ± 0.83	5.55 ± 1.12	1.91 ± 0.06^{a}	1.87 ± 0.28^{a}	2.12 ± 0.05^{a}	3.19 ± 0.32^{b}
2-Penten-1-ol, (Z)-	1325	1324	0.84 ± 0.02^{a}	0.49 ± 0.03^{a}	0.88 ± 0.07^{a}	1.47 ± 0.23^{b}	1.06 ± 0.18^{b}	1.09 ± 0.17^{b}	1.36 ± 0.06^{b}	1.89 ± 0.39^{a}	0.22 ± 0.06^{a}	0.38 ± 0.09^{a}	0.25 ± 0.00^{a}	0.78 ± 0.03^{b}
1-Hexanol	1359	1358	34.21 ± 7.11^{b}	34.67 ± 4.38^{b}	25.71 ± 2.46^{a}	7.69 ± 0.10^{a}	9.05 ± 3.19	7.67 ± 1.07	7.97 ± 1.14	6.74 ± 1.79	7.05 ± 1.66^{ab}	7.60 ± 1.13^{b}	4.22 ± 0.45^{ab}	3.86 ± 0.11^{a}
3-Hexen-1-ol, (Z)-	1387	1387	ND	ND	ND	ND	0.12 ± 0.03	0.1 ± 0.01	0.18 ± 0.02	0.11 ± 0.02	ND	ND	ND	ND
3-Heptanol, 6-methyl-	1398	1397	ND	ND	ND	ND	0.16 ± 0.14^{b}	0.12 ± 0.01^{ab}	0.10 ± 0.01^{a}	0.12 ± 0.01^{ab}	ND	ND	ND	ND
1-Octen-3-ol	1456	1454	4.79 ± 0.70	5.08 ± 1.63	3.09 ± 0.59	4.51 ± 0.69	40.30 ± 14.09	57.11 ± 13.83	50.23 ± 7.92	42.40 ± 22.21	3.51 ± 0.13	6.40 ± 0.82	4.27 ± 0.66	4.81 ± 0.61
1-Heptanol	1461	1461	1.01 ± 0.10^{a}	1.08 ± 0.14^{a}	0.59 ± 0.06^{b}	0.51 ± 0.10^{a}	0.47 ± 0.19	0.43 ± 0.11	0.43 ± 0.03	0.28 ± 0.10	1.02 ± 0.10^{ab}	1.07 ± 0.19^{b}	0.64 ± 0.13^{a}	0.79 ± 0.11^{ab}
1-Hexanol, 2-ethyl-	1497	1494	0.94 ± 0.12	2.22 ± 0.70	1.92 ± 0.13	1.78 ± 0.63	0.32 ± 0.07	0.25 ± 0.02	0.36 ± 0.01	0.26 ± 0.06	4.01 ± 0.91	4.04 ± 1.20	5.36 ± 1.01	2.99 ± 0.45
2-Hepten-1-ol, (E)-	1517	1516	ND	ND	ND	ND	0.1 ± 0.01^{a}	ND	ND	ND	ND	ND	ND	ND
1-Octanol	1558	1564	1.46 ± 0.36	1.82 ± 0.50	1.88 ± 0.03	1.26 ± 0.11	0.4 ± 0.13^{b}	0.39 ± 0.08^{b}	0.32 ± 0.01^{ab}	0.24 ± 0.05^{a}	1.90 ± 0.23^{b}	1.70 ± 0.19^{ab}	1.15 ± 0.07^{a}	1.78 ± 0.16^{b}
3-Cyclohexen-1-ol, 1-methyl-4-(1-methylethyl)-	1582	1585	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.07 ± 0.19	ND
2-Octen-1-ol, (E)-	1626	1631	ND	ND	ND	ND	5.64 ± 1.58	7.87 ± 1.63	6.17 ± 0.90	7.99 ± 1.23	ND	ND	ND	ND
1-Nonanol	1666	1668	ND	ND	ND	ND	22.05 ± 5.07^{b}	0.11 ± 0.02^{a}	1.45 ± 0.19^{a}	0.04 ± 0.01^{a}	0.83 ± 0.25^{a}	0.51 ± 0.13^{a}	ND	ND
Aldehydes (9)														
Butanal, 2-methyl-	910	909	ND	ND	ND	ND	0.11 ± 0.02^{a}	ND	ND	0.35 ± 0.01^{b}	ND	ND	ND	6.30 ± 1.02^{a}
Butanal, 3-methyl-	908	913	ND	ND	ND	ND	ND	ND	ND	0.69 ± 0.11^{a}	ND	ND	ND	7.72 ± 1.33^{a}
Hexanal	1078	1073	2.45 ± 0.57^{a}	1.53 ± 0.20^{a}	15.8 ± 1.62^{b}	28.94 ± 2.44^{c}	2.73 ± 0.24^{ab}	2.83 ± 0.11^{a}	2.73 ± 0.15^{ab}	4.89 ± 0.52^{b}	1.92 ± 0.18^{a}	1.97 ± 0.28^{a}	1.68 ± 0.20^{a}	4.55 ± 0.66^{b}
Heptanal	1182	1183	1.89 ± 0.38^{ab}	2.07 ± 0.19^{b}	0.88 ± 0.09^{a}	0.87 ± 0.25^{a}	0.16 ± 0.03^{a}	0.25 ± 0.09^{ab}	0.16 ± 0.03^{a}	0.33 ± 0.10^{b}	1.07 ± 0.29^{a}	1.00 ± 0.22^{a}	0.44 ± 0.02^{a}	1.88 ± 0.24^{b}
Octanal	1291	1286	0.75 ± 0.07^{a}	1.84 ± 0.38^{b}	0.58 ± 0.07^{a}	0.37 ± 0.06^{a}	0.14 ± 0.03^{ab}	0.19 ± 0.05^{b}	0.10 ± 0.01^{a}	0.13 ± 0.01^{ab}	0.94 ± 0.25	1.07 ± 0.13	0.56 ± 0.08	1.20 ± 0.41
Nonanal	1397	1392	4.15 ± 0.30^{b}	4.55 ± 0.41^{b}	1.71 ± 0.26^{a}	2.61 ± 0.55^{a}	0.39 ± 0.10^{bc}	0.11 ± 0.02^{a}	0.32 ± 0.09^{b}	0.55 ± 0.12^{a}	3.35 ± 0.67^{a}	3.35 ± 0.43^{a}	2.19 ± 0.46^{a}	5.17 ± 0.98^{b}
2-Octenal, (E)-	1413	1429	ND	ND	ND	ND	0.85 ± 0.08	1.13 ± 0.24	0.86 ± 0.07	0.74 ± 0.16	ND	ND	ND	ND
Benzaldehyde	1520	1525	ND	ND	ND	ND	0.24 ± 0.03^{a}	0.59 ± 0.04^{a}	0.24^{a}	7.51 ± 0.57^{b}	0.64 ± 0.09^{a}	0.52 ± 0.06^{a}	0.45 ± 0.04^{a}	1.74 ± 0.33^{b}
2-Nonenal, (E)-	1538	1540	ND	ND	ND	ND	0.1 ± 0.01^{b}	0.13 ± 0.03^{b}	ND	0.06 ± 0.01a	ND	ND	ND	ND

Values are means ± standard deviations of three individual samples. Mean values with different superscript across the column are significantly different (p> 0.05).

ND, Not detected; RIL, retention index; RIC, retention indices and their mass spectra with NIST mass spectral library.

Table 3.2 (continued)

Compound name by class	RIL	RIC	Muscle				Gill				Skin			
			0	3	6	9	0	3	6	9	0	3	6	9
Ketones (8)														
2-Butanone	904	900	0.49 ± 0.08^{a}	0.47 ± 0.08^{a}	ND	ND	0.14 ± 0.02^{b}	0.15 ± 0.03^{b}	ND	0.09 ± 0.01^{a}	ND	ND	ND	ND
3-Pentanone	974	971	0.83 ± 0.01^{a}	1.49 ± 0.07^{a}	0.81 ± 0.11^{a}	3.15 ± 0.32^{b}	0.76 ± 0.07	0.71 ± 0.08	0.98 ± 0.03	3.07 ± 1.06	ND	ND	ND	ND
2,3-Pentanedione	1054	1053	0.78 ± 0.12	0.63 ± 0.02	1.03 ± 0.19	1.05 ± 0.14	0.11 ± 0.11^{a}	0.14 ± 0.01^{a}	0.23 ± 0.03^{ab}	0.37 ± 0.05^{b}	ND	ND	ND	ND
2-Heptanone	1184	1181	0.27 ± 0.03	0.35 ± 0.01	0.37 ± 0.03	0.28 ± 0.06	0.18 ± 0.08	0.13 ± 0.01	0.15 ± 0.01	0.16 ± 0.03	ND	ND	ND	ND
2-Heptanone, 6-methyl-	1237	1236	ND	ND	ND	ND	0.15 ± 0.04^{a}	0.12 ± 0.02^{a}	ND	ND	ND	ND	ND	ND
3-Octanone	1253	1253	0.26 ± 0.01^{a}	0.26 ± 0.06^{a}	ND	0.31 ± 0.02^{a}	1.20 ± 0.47^{ab}	1.18 ± 0.19^{ab}	0.96 ± 0.10^{a}	1.55 ± 0.29^{b}	0.23 ± 0.05^{a}	0.32 ± 0.04^{b}	ND	0.31 ± 0.01^{b}
Cyclohexanone	1285	1282	1.9 ± 0.13^{a}	2.12 ± 0.42^{a}	2.55 ± 0.19^{a}	3.34 ± 0.54^{b}	0.22 ± 0.05^{a}	0.3 ± 0.04^{a}	0.44 ± 0.02^{b}	0.55 ± 0.12^{b}	2.15 ± 0.15^{a}	3.45 ± 0.29^{b}	3.66 ± 0.16^{b}	6.91 ± 0.14^{c}
1-Hepten-3-one	1298	1298	ND	ND	ND	ND	1.25 ± 0.23	1.16 ± 0.00	1.09 ± 0.18	0.75 ± 0.07	ND	ND	ND	ND
Furans (2)														
Furan, 2-ethyl-	944	946	ND	ND	ND	ND	0.42 ± 0.14^{a}	0.61 ± 0.09^{b}	0.37 ± 0.03^{a}	0.55 ± 0.19^{ab}	ND	ND	ND	ND
Furan, 2-pentyl-	1231	1230	ND	ND	ND	ND	0.13 ± 0.02	0.14 ± 0.02	0.11 ± 0.02	0.13 ± 0.01	ND	ND	ND	ND
Acid (1)														
Butyrolactone (Butanoic acid, 4-hydroxy-)	1635	1637	1.21 ± 0.09^{b}	0.35 ± 0.08^{a}	0.41 ± 0.07^{a}	1.08 ± 0.11^{b}	0.18 ± 0.10	0.07 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	1.66 ± 0.14^{b}	0.54 ± 0.01^{a}	0.57 ± 0.04^{a}	0.90 ± 0.13^{a}
Ester (1)														
Hexanoic acid, ethyl ester	1233	1234	ND	ND	ND	ND	0.11 ± 0.04	0.09 ± 0.01	0.06 ± 0.01	0.13 ± 0.02	ND	ND	ND	ND
Hydrocarbons (5)														
2,4-Octadiene	925	925	ND	ND	ND	ND	ND	0.07 ± 0.00^{a}	ND	0.20 ± 0.05^{b}	ND	ND	ND	ND
Decane	1000	999	0.76 ± 0.08^{a}	ND	ND	ND	0.1 ± 0.01^{a}	0.09 ± 0.00^{b}	0.2 ± 0.02^{a}	0.21 ± 0.04^{b}	5.32 ± 0.54^{b}	2.10 ± 0.42^{a}	2.52 ± 0.15^{a}	4.67 ± 0.97^{b}
1,3-trans,5-cis-Octatriene	1097	1096	ND	ND	ND	ND	0.34 ± 0.07^{a}	0.36 ± 0.05^{a}	0.58 ± 0.06^{b}	0.31 ± 0.10^{a}	ND	ND	ND	ND
Dodecane	1120	1197	ND	ND	ND	ND	ND	ND	ND	ND	1.96 ± 0.36^{a}	1.01 ± 0.13^{a}	1.14 ± 0.12^{a}	3.63 ± 0.89^{b}
Pentadecane	1500	1498	0.53 ± 0.06^{a}	1.46 ± 0.23^{b}	4.92 ± 0.28^{d}	3.18 ± 0.51^{c}	0.21 ± 0.03^{a}	0.29 ± 0.08^{a}	0.90 ± 0.10^{b}	0.34 ± 0.08^{a}	1.13 ± 0.17^{a}	2.51 ± 0.51^{a}	6.74 ± 0.57^{b}	8.37 ± 1.42^{b}
Aromatic compounds (6)														
Benzene	938	932	ND	ND	ND	ND	0.38 ± 0.10	0.27 ± 0.08	0.34 ± 0.04	0.34 ± 0.07	ND	ND	ND	ND
Trichloromethane	1015	1015	29.17 ± 4.29	25.42 ± 8.57	16.49 ± 2.06	22.56 ± 11.86	0.50 ± 0.14^{a}	4.43 ± 1.06^{b}	2.86 ± 0.23^{ab}	0.93 ± 0.10^{a}	46.85 ± 3.01^{b}	44.38 ± 0.15^{b}	41.62 ± 10.87^{b}	3.68 ± 0.54^{a}
Toluene	1037	1026	4.43 ± 0.79^{a}	3.65 ± 0.18^{a}	10.87 ± 2.26^{b}	5.14 ± 0.62^{a}	1.17 ± 0.34^{ab}	0.46 ± 0.05^{a}	3.32 ± 1.06^{ab}	3.37 ± 1.04^{b}	7.91 ± 0.50^{a}	5.57 ± 0.30^{a}	14.34 ± 2.76^{b}	9.14 ± 0.65^{a}
Styrene	1256	1251	0.14 ± 0.03^{a}	0.52 ± 0.05^{bc}	0.75 ± 0.17^{c}	0.27 ± 0.01^{ab}	ND	ND	ND	ND	0.25 ± 0.02^{a}	1.1 ± 0.16^{b}	1.21 ± 0.19^{b}	1.02 ± 0.03^{b}
Benzene, 1,2,4-trimethyl-	1275	1274	ND	ND	ND	ND	ND	ND	0.04 ± 0.00^{a}	0.20 ± 0.00^{b}	0.32 ± 0.02^{a}	0.42 ± 0.05^{a}	0.30 ± 0.01^{a}	1.13 ± 0.29^{b}
Benzene, 1,4-dichloro-	1446	1443	ND	ND	ND	ND	ND	ND	ND	ND	1.18 ± 0.06^{a}	0.92 ± 0.15^{a}	1.09 ± 0.21^{a}	ND

Values are means ± standard deviations of three individual samples. Mean values with different superscript across the column are significantly different ($p > 0.05$).

ND, Not detected; RIL, retention index; RIC, retention indices and their mass spectra with NIST mass spectral library.

Aldehydes are essential compounds giving characteristic odors, both pleasant and unpleasant, to foods. Their odor threshold values are typically lower than those of alcohols. As a result, even trace levels of aldehydes have the potential to dominate flavor of other compounds (Wang et al., 2019). 3-Methylbutanal and benzaldehyde were detected in gill and skin with the highest concentration at day 9. They are likely to produce from Strecker degradation of leucine and phenyl glycine, respectively (Mu et al., 2017). The predominant aldehydes found in tilapia tissues are hexanal, heptanal, and nonanal. Hexanal was mostly derived from the oxidation of linoleic acid (Kawai, 1996) and related to degradation of volatile compounds namely (E)-2-octenal (Josephson and Lindsay, 1987; Koelsch, Downes, and Labuza, 2006), and the levels of lipid oxidation and flavor production were reflected by its contents. Our findings are remarkably similar to those of Miyasaki, Hamaguchi, and Yokoyama (2011) who reported that hexanal in fish meat was detected early on and that the concentration of hexanal rapidly increased during ice storage. 3-Methylbutanal and hexanal both contribute to the smell of rabbit fish skin, muscle, viscera, and stomach contents (Jiarpinijnun et al, 2022). Heptanal and nonanal are generated from the oxidation of oleic acid and linoleic acid. Heptanal is the major volatile compound detected in the Asian seabass muscle and has been provided as an indicator of flavor deteriorations for fish products (Maqsood and Benjakul, 2011; Augustin, Sanguansri, and Bode, 2006). Another detected compound, (E)-2-octenal and (E)-2-nonenal which are produced by LOX on arachidonic acid. Nonanal, octanal, and (E) -2-octenal were the most powerful contributors to the fishy odor of tilapia (Liu, Hu, and Li, 2014).

The main ketone identified in our study is cyclohexanone. Enzymatic degradation of polyunsaturated fatty acids, amino acids, or microbial oxidation generate ketones (Survey, Pan, and Sahidi, 1998). Gill had the highest concentrations of ketone compounds, followed by the muscles and skin. 2,3-Pentanedione was identified in gill and muscle. Iglesias and Medida (2008) reported that 2,3-Pentanedione as an indicator of lipid oxidation in chill fish muscle.

Furan compounds were detected and identified only in the gill of tilapia including 2-ethyl furan and 2-pentyl furan. Furans and their derivatives could be produced through the oxidation of fatty acids. A mechanism for the formation of 2-ethylfuran in fish muscle has previously been proposed (Medina et al., 1999), including

omega-3 fatty acid β-oxidation to generate conjugated dienes radicals, which can react with oxygen to produce vinyl hydroperoxides; cleavage of vinyl hydroperoxide results in the formation of an alkoxyl radical, which proceeds cyclization, to form 2-ethylfuran (Medina et al., 1999). 2-Ethylfuran was detected to be the highest concentration after 3 days of ice storage. 2-Ethylfuran has been reported previously in hilsa and was found to be highest after ice storage of 168 h, indicating that the unfavorable fishy odor increased during storage due to lipid oxidation (Ganguly, Mahanty, Mitra, Raman, and Mohanty 2017; Leduc et al., 2012). 2-Ethylfuran has been considered to contribute to the distinctive sardine-fishy odor (Prost et al., 2004). Besides 2-ethylfuran, 2-pentylfuran is a well-known autoxidation product from linoleic acid and has low threshold values (Giri et al., 2010). 2-Ethylfuran and 2-pentylfuran were the main contributors to the aromatic flavor of the short-necked clam hydrolysate (Chen et al., 2016).

Hydrocarbons and aromatic compounds were also identified in our study. Although their presence has been reported in a variety of fish, the origin of these compounds has not been fully elucidated (Ganguly et al., 2017; Iglesias and Meida, 2008; Wang et al., 2019). Hydrocarbons had high sensory thresholds, thus they contribute less to the overall odor formation of fish (Zhou, Chong, Ding, Gu, and Liu, 2016).

3.4.4 Principal component Analysis (PCA)

In muscle, the first two components of PCA explained 65.35% of the variation (Figure 3.4). According to this PCA, volatile compounds were grouped by storage time. 2-Butanone, hexanal, 1-butanol, 1-penten-3-ol, heptanal, nonanal, pentadecane were closely related to PC1 while 3-methyl-1-butanol, 3-pentanone, 3-octanone, 1-heptanol were closely related to PC2. 2-Butanone and nonanal are the major volatile compounds present in 0 day. 2-Butanone was detected in day 0 and and disappeared after 3 day of storages, while nonanal was detected and its quantities decreased with increasing time period of storage. Nonanal contributes to fatty, green, fish-like odors and is found in some fish species, especially freshwater species (Jones et al., 2022). Thus, 2-butanone and nonanal can be considered a freshness indicators of tilapia muscle. 1-Penten-3-ol, hexanal and 3-methyl-1-butanol are the major volatile compounds present in day 9, which showed positive correlation and their relative quantities increased with increasing time storage ($p < 0.05$). 1-Penten-3-ol is established

when lipoxygenase reacts with EPA and hydroperoxide lyases which is used as a marker of lipid oxidation in Atlantic horse mackerel (Iglesias and Medina, 2008). Linoleic acid was primarily oxidized to produce hexanal (Kawai, 1996). According to Miyasaki, Hamaguchi, and Yokoyama (2011), hexanal was detected early in fish meat, and its concentration increased rapidly during ice storage. 3-Methyl-1-butanol has an offensive odor and is derived from the oxidative deamination of leucine by bacteria predominately responsible for spoilage (Wang, Chen, Zhang, Wang, and Shi, 2019). In this study, these compounds were potential markers for measuring the degree of lipid oxidation in tilapia muscle.

In gill, the principle component 1(PC1) explained 37.62% and principle component 2 (PC2) explained 20.28% of total variance (Figure 3.5). According to this PCA, volatile compounds were grouped according to storage time, which appeared in three quadrants: 6 days; 0 day and 3 days; 9 days. The volatile compounds 2-methyl-butanal, 3-methyl-butanal, 2,4-octadiene, 3-pentanone, decane, 2,3-pentanedione, hexanal, heptanal, 6-methyl-2-heptanone, 3-octnone, 1-pentanol, 1,2,4-trimethyl benzene, cyclohexanone, 2-penten-1-ol, nonanal, 1-haptanol, benzaldehyde, 1-octanol were closely related to PC1 while 2-butanone, 1,3-trans,5-cis-octatriene, 1-penten-3-ol, 3-methyl-1-butanol, 2-pentyl-furan, octanal, 3-hexen-1-ol, 6-methyl-3-heptanol, pentadecane, 2-nonenal were closely related to PC2. The most abundant volatile compounds in the third quadrant are 6-methyl-2-heptanone and 2-nonenal (0 day and 3 days). These volatiles were detected between 0 and 3 days and were no longer detectable after 3 days of storage. 2-Nonanal is generated by LOX on arachidonic acid and contributes to tilapia's fishy odor (Qian, Fei, and Fan, 2012). Therefore, these can be regarded as indicators of the freshness of tilapia gill. 3-Methyl-butanal, 2,4-octadiene and hexanal are the major volatiles present in day 9 which showed positive correlation with each other. As a result, these volatiles could be used to track the lipid oxidation of tilapia gill during ice storage.

In skin, the first two components of PCA explained 74.69 % of the variation (Figure 3.6). According to this PCA, volatile compounds were grouped by storage time.2-Methylbutanal, 3-methylbutanal, hexanal, 1-penten-3-ol, heptanal, 3-methyl-1-butanol, 1-pentanol, cyclohexanone, 2-penten-1-ol, nonanal, benzaldehyde were closely related to PC1. While 3-octanone, 1-heptanol, 1-octanol, 3-cyclohexen-1-ol,

butyrolactone, 1-nonanol were closely related to PC2. The PCA biplot revealed the correlation between various volatile compounds and also explains the overall variation in the data sets. 1-Heptanol and 1-nonanol are the major volatile compounds present in day 0 and 3. These two compounds detected in day 0 and their relative quantities decreased as ice storage time increased. While 1-nonanol disappeared after 3 days of storage, suggesting it as freshness indicator for tilapia skin. 2-Methylbutanal, 3-methylbutanal, hexanal, 1-penten-3-ol, heptanal, 3-methyl-1-butanol,2-penten-1-ol, and nonanal are the major volatiles, showing in day 9. 2-Methylbutanal and 3-methylbutanal were detected at 9 days of storage and they are produced from Strecker degradation of leucine (Mu et al, 2017). Hexanal, 1-penten-3-ol, heptanal, 3-methyl-1-butanol,2-penten-1-ol, heptanal and nonanal appeared after 0 day of storage, and their amount increased with storage time.

The first two components of PCA explained 65.64% of the variation (Figure 3.7). The muscle, gill and skin are clearly separated in different quadrants. The skin sample was characterized by peroxide values and the main volatile compounds, namely 1-3-methyl butanol, and 2-methyl butanal, which increased with the storage time. It should be noted that the effect of storage time on the measured parameters is well correlated for the skin sample. Our study suggests that the skin should be a target tissue for monitoring freshness quality of tilapia during ice storage. The gill was characterized by LOX activity, PUFA contents, and volatile compounds, particularly furan, 2-pentyl-, 2-octenal, 3-heptanol, 6 methyl-, and 2-octen-1-ol. The skin and muscle samples are positioned in the opposite PCA quadrant from the gill sample, indicating lower levels of PUFA contents, LOX activity, and associated volatiles in muscle. Our study indicates that gill is the main tissue of lipid oxidation induced by LOX during ice storage. Furan, 2-pentyl-, 3-heptanol, 6-methyl-, 2-octenal, (E)-, 1-octen-3-ol and 2-octen-1-ol are the main volatile compounds in tilapia gill.

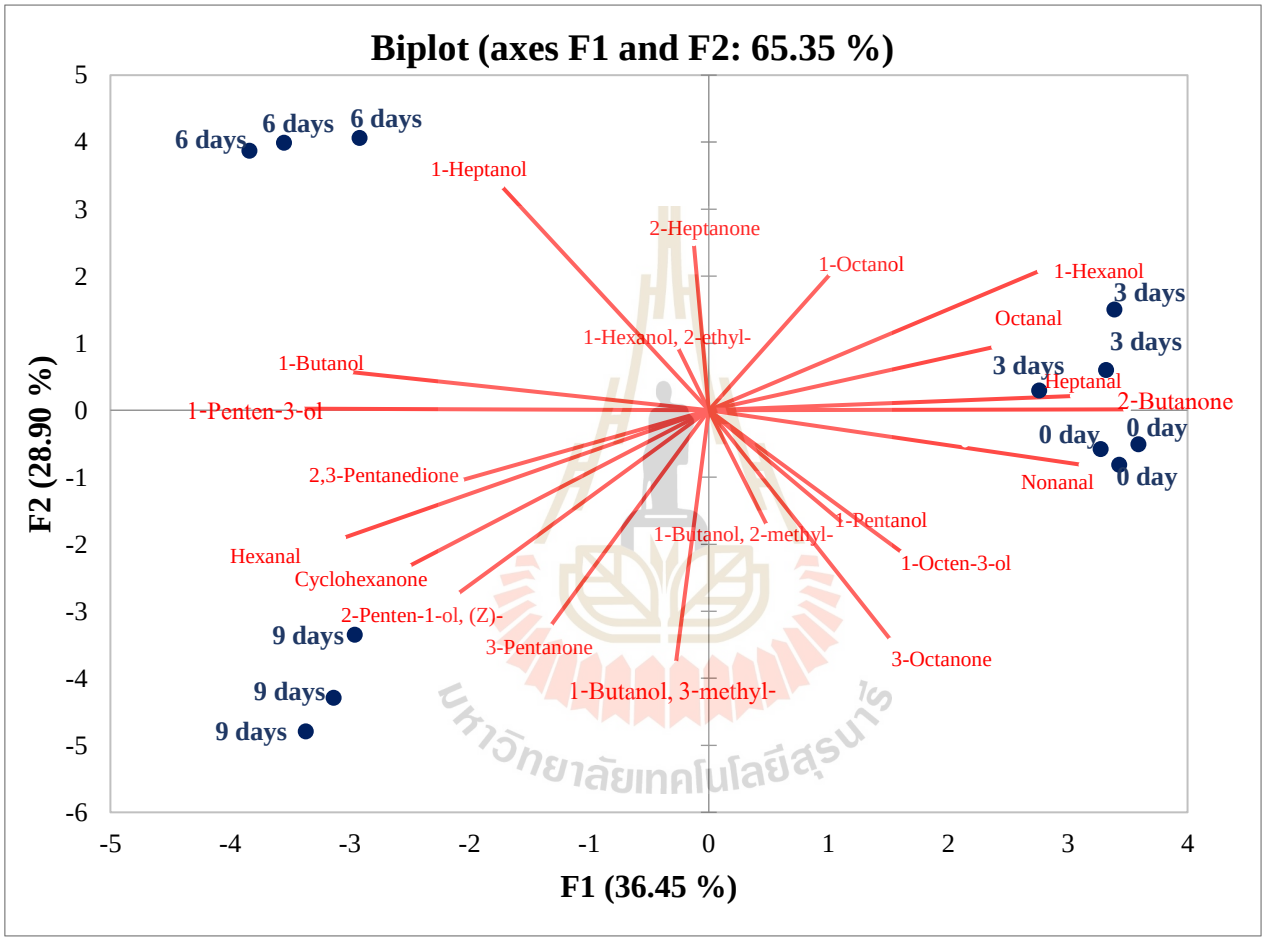

Figure 3.4 Principal component analysis (PCA) biplot of volatile compounds present in muscle of tilapia in different storage times assessed by SPME-GC/MS.

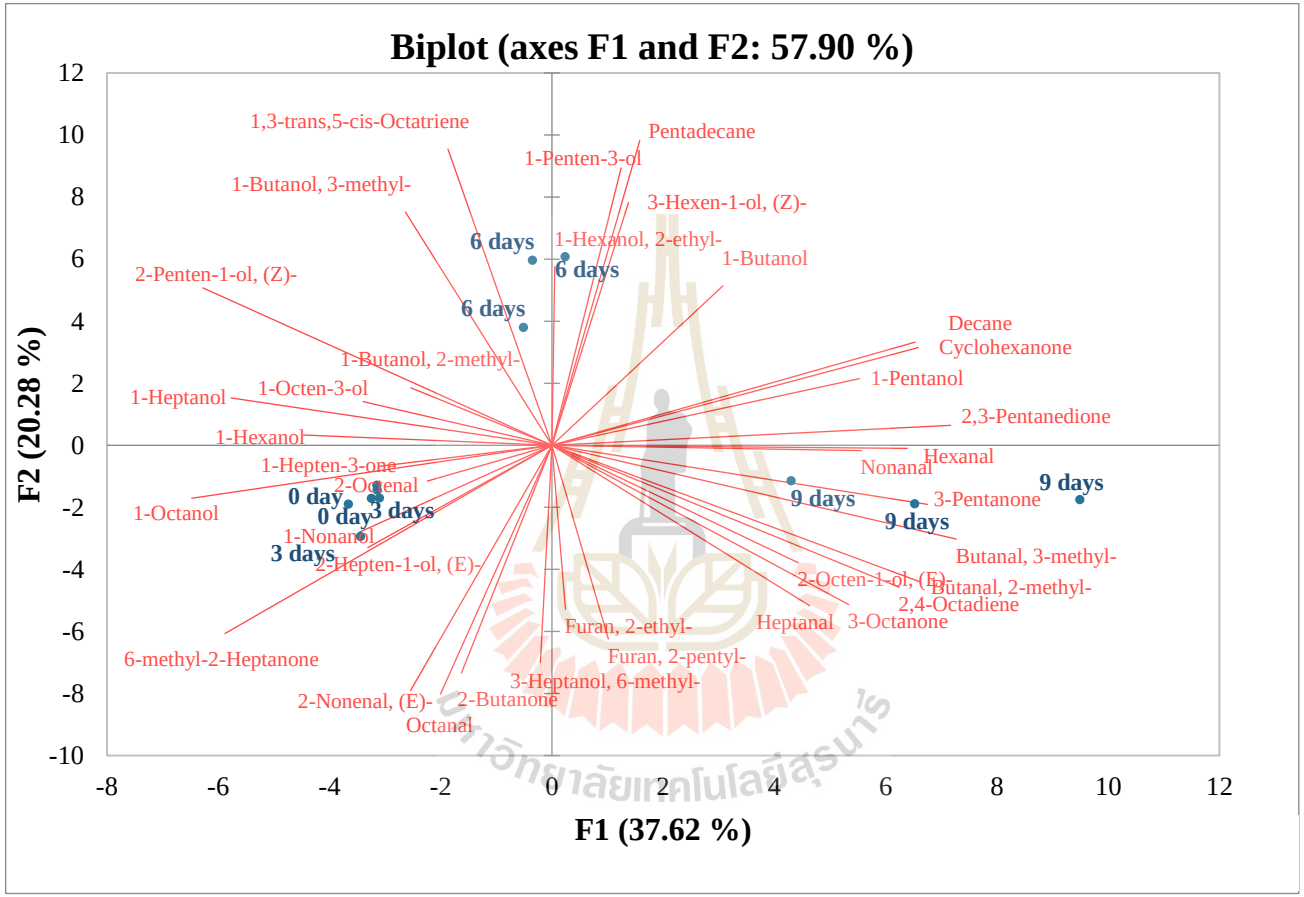

Figure 3.5 Principal component analysis (PCA) biplot of volatile compounds present in gill of tilapia in different storage times assessed by SPME-GC/MS.

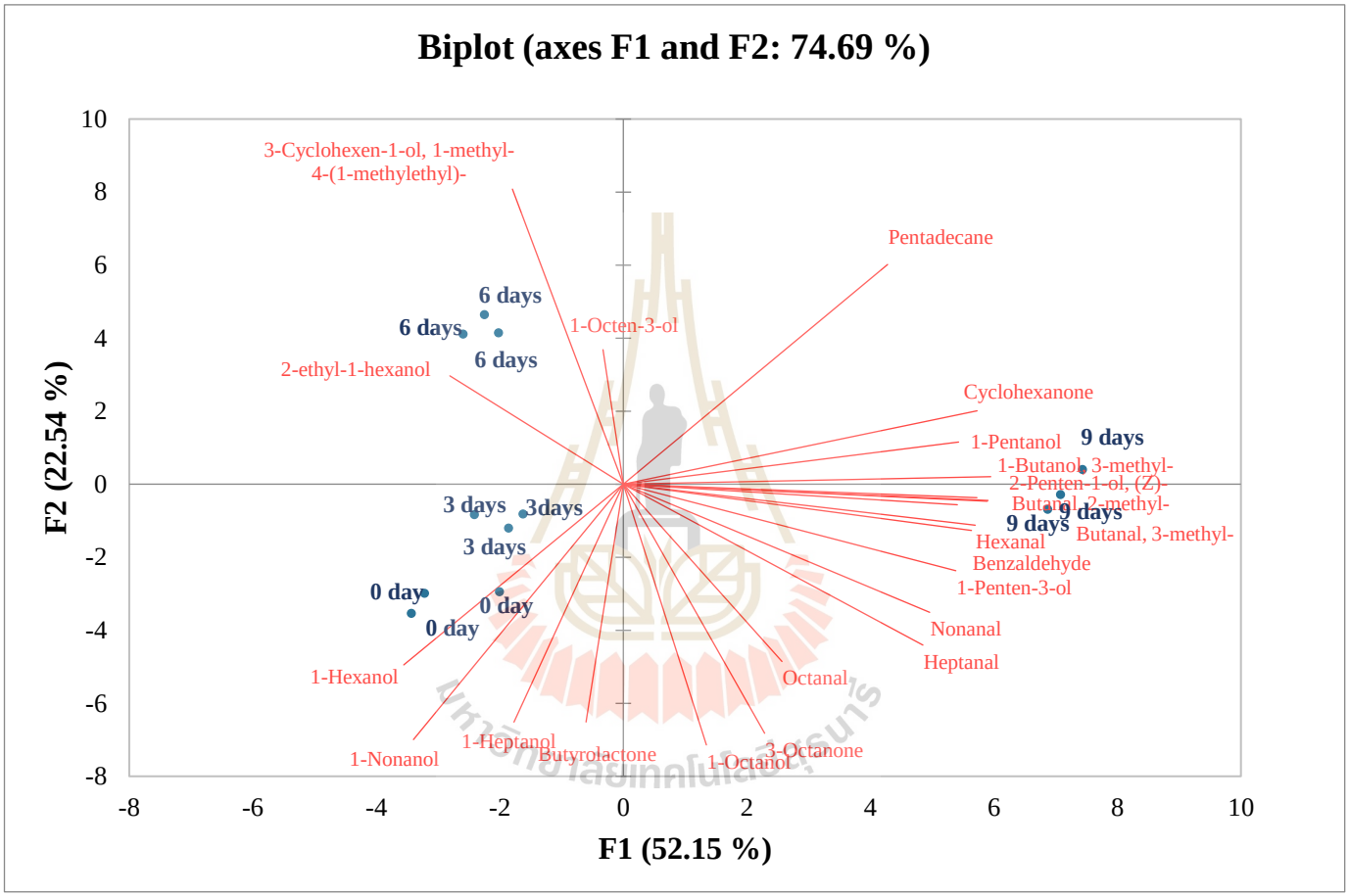

Figure 3.6 Principal component analysis (PCA) biplot of volatile compounds present in skin of tilapia in different storage times assessed by SPME-GC/MS.

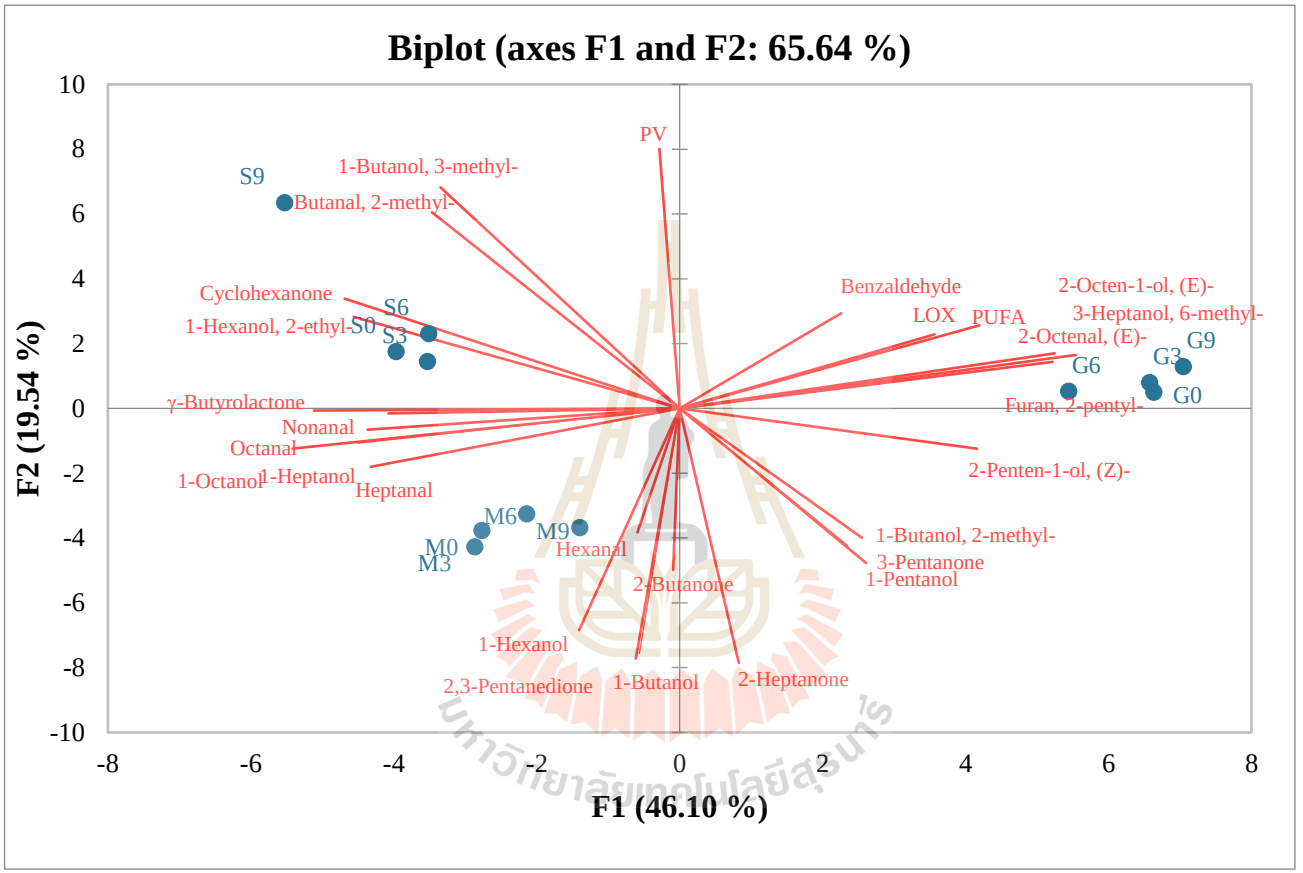

Figure 3.7 Principal component analysis (PCA) biplot describing measured quality parameters namely volatile compounds, peroxide values, lipoxygenase activity, and PUFA of muscle (M), gill (G), and skin (S) of tilapia stored in ice at different times 0, 3, 6 and 9 days. Each point is the average of three measurements of samples from a batch of three fishes. LOX, lipoxygenase activity; PV, peroxide value; PUFA, polyunsaturated fatty acids.

3.5 Conclusions

Lipid oxidation of tilapia differed among body parts. During prolonged ice storage, the gill of tilapia was the most prone to lipid oxidation. Free fatty acid composition decreased as the storage time was extended. The activity of LOX was relatively high during storage and LOX could catalyze oxidation of free fatty acids, suggesting that it was an important enzyme to promote volatile substances. The majority of volatile compounds exhibited statistically significant differences during ice storage when compared to their initial values, indicating that ice storage affected the volatile nature of tilapia. According to the PCA, 2-butanone and nonanal in muscle, 6-methyl-2-haptanone and 2-nonanal in gill, and 1-haptanol, and 1-nonanol in skin have the potential to be used as freshness indicators and hexanal was used to track the lipid oxidation of tilapia during ice storage. To preserve the quality of tilapia, gills should be removed prior to ice storage and filleting.

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มหาวิทยาลัยเทคโนโลยีสุรนารี

CHAPTER IV
STUDY ON LIPOXYGENASE ACTIVTY IN TROFICAL FISH AND SURIMI: PARTIAL PURIFICATION AND CHARACTERIZATION OF LIZARDFISH (*Saurida tumbil*) GILL

4.1 Abstract

LOX activity of major tropical fish used for surimi production, including threadfin bream, lizardfish, and goatfish were followed in gill, skin, and muscle. The highest LOX activity of 376.56 U/mg protein was found in lizardfish gill, whereas the highest LOX activity in skin and muscle was found in threadfin bream of 60.67 and 137.04 U/mg protein, respectively. Among tropical fish, threadfin bream showed the highest amount of peroxide value. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the main polyunsaturated fatty acids (PUFA) in all tissues. Lizardfish surimi contained high amount of DHA and EPA. Residual LOX activity of 57.85 U/g sample remained after washing, indicating that LOX would contribute to oxidative off-flavor of surimi. LOX was purified from lizardfish gill was purified by two successive chromatographic steps of Sephacryl S-200 and DEAE-Sepharose, resulting in 3.52 % yield and 22.43-fold increase in purity. Optimum activity was found at 25 ° C and pH 7.5 with pH stability at 6.0 - 8.5. At 15 ° C, residual LOX activity remained 80%, suggesting that the enzyme might contribute to fish lipid oxidation during refrigerated storage. The enzyme was thermally inactivated at 50 ° C. The most preference substrate was 2.5 mM EPA. The enzyme was inhibited by 1 mM ethylenediaminetetraacetic acid (EDTA) and activated by 1 mM Fe^{2+}, Na^{2+}, and Ca^{2+}.

Keywords: Lipoxygenase, Purification, Lizardfish, Lipid oxidation, Surimi

4.2 Introduction

Oxidation of lipids deteriorates fish quality, resulting in off-odors/flavors, color problems, texture defects, and safety concerns (Wu, Richards, and Undeland, 2022). Fish contain high amounts of omega-3 and omega-6 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic (DHA), eicosapentaenoic acids (EPA), linoleic acid (LA), and alpha-linolenic acid (ALA) (Strobel, Jahreis, and Kuhnt, 2012). Lipoxygenase (LOX, 1inoleate: oxygen oxidoreductase, EC 1.13.11.12), is a dioxygenase that oxygenates polyunsaturated fatty acids (PUFA) containing a cis, cis- 1,4 pentadiene structure to form conjugated unsaturated fatty acid hydroperoxides (Grechkin, 1998). Hydroperoxy fatty acids can be further metabolized to produce alcohols, aldehydes, ketones and hydrocarbons, lowering fish quality (Josephson et al., 1983). Decanal, nonanal, octanal, and (E)-2-nonenal, exhibiting green and fatty note, were the most potent contributors to the aroma of the raw tuna fish (Zhang, Ma, and Dai, 2019). Iglesias et al. (2009) reported that Z-4-heptenal is an important contributor to off-flavors in gilthead sea bream, which is produced by the LOX-catalyzed oxidation of EPA. Moreover, strong fishy odor of silver carp is caused by 2, 4-heptadienal (E, E) resulted from LA oxidation catalyzed by LOX (Fu et al., 2009). LOX has been studied in muscle, skin, and gill tissue of various aquatic species, including trout, rockfish, mackerel, gray mullet, grass carp, sardine (German and Creveling, 1990; Banerjee, Khokhar, and Apenten, 2002; Hong, Shim, and Byun, 1994; Hsu and Pan, 1996; Wang et al., 2012; Mohri, Cho, Endo, and Fujimoto, 1992). However, few reports have been studied on LOX in tropical fish used for surimi production.

Goatfish (*Mullidae spp.*), lizardfish (*Saurida spp.*), and threadfin bream (*Nemipteridae spp*.) are raw materials for tropical surimi production. Typically, surimi has bland taste and less fishy note than its respective mince. However, tropical surimi is known to have strong fishy odor and sometime is considered as “off-odor”. This is likely due to poor post-harvest handlings of raw materials. Although washing can remove odorous compounds, the extent of strong off-note is much higher than cold water species surimi (An, Qian, Alcazar Magana, Xiong, and Qian, 2020). Membrane phospholipids rich in PUFA are difficult to remove by washing and are very sensitive to oxidation (Eymard, Baron, and Jacobsen, 2009). These phospholipids can serve as a

substrate of LOX, causing off-flavors. However, it is not known how much LOX residual activity remains after washing. Understanding LOX characteristics of tropical fish would lead to better control strategies for surimi qualities. Therefore, the objectives from this study were to prepare a partially purified and characterize LOX from lizardfish gill. The effects of industrial surimi washing process on LOX activity and fatty acid composition were also studied.

4.3 Materials and methods

4.3.1 Chemicals and reagents

Bovine serum albumin (BSA), 2-thiobarbituric acid, cumene hydroperoxide, guanidine hydrochloride, trichloroacetic and ferrous chloride were purchased from Fluka (Buchs, Switzerland). Diethylaminoethyl (DEAE)-Sepharose and Sephacryl-200 were purchased from GE Healthare (Uppsala, Sweden). Oleic acid (≥98%), Arachidonic acid (≥99%), Linolenic acid (≥99%), Eicosapentaenoic acid (EPA) (≥99%), Docosahexaenoic acid (DHA) (≥99%), heptadecanoic acid (≥99%), tween-20, L-glutathione reduced (≥98.0) and cyclohexanol were purchased from Sigma-Aldrich (Oakville, Canada). Other chemicals and reagents were of analytical grade.

4.3.2 Sample preparation

Goatfish (*Mullidae spp.*), lizardfish (*Saurida spp.*), and threadfin bream (*Nemipteridae spp.*) were obtained from Andaman Surimi Industries company (Samut Sakhon, Thailand). Fish were caught and off-loaded approximately 7-10 days after capture. Fish were placed in ice with a fish/ice ratio of 1:2 (w/w) and transported to Suranaree University of Technology within 5 h. Whole fish were immediately washed and kept in ice with a fish/ice ratio of 1:2 (w/w). Skins, gill, and flesh were manually separated and immediately stored at -70 $^{\circ}$C until use. Mince fish, the first washed mince, the second washed mince, the third washed mince, mince from refiner, screw press and surimi of all 3 species were also collected at the surimi production facility of Andaman Surimi Industries. Surimi samples were either used immediately or stored at -70 $^{\circ}$C until use.

4.3.3 Total lipid content

Total lipids content of gill, skin, and muscle of 3 tropical fish and surimi samples were analyzed according to Bligh & Dyer method (Bligh and Dyer, 1959). Samples (12 g) was homogenized with 60 ml chloroform and methanol solution (2:1, v/v) at 8000 rpm for 2 min at 4 ° C using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). The homogenates were filtered through Whatman No. 1 filter paper into a separating funnel. Thereafter, 24 ml of chloroform, 24 ml of distilled water and 0.58% (v/v) sodium chloride solution was added. The mixture was gently shaken. After separation, chloroform phase was drained off into a 125 ml Erlenmeyer flask containing about 2–5 g of anhydrous sodium sulfate and was shaken well to remove any traces of water that might be present in the chloroform phase. The solution was filtered through Whatman No. 1 filter paper into a pre-weighed 125 ml Erlenmeyer flask and solvent was removed by flushing with nitrogen. To allow for further analysis of phospholipids and fatty acid, the extracted lipid was dissolved in 10 mL chloroform and stored at -80 ° C.

4.3.4 Fatty acid profile

Fatty acid profiles of extracted muscle were determined as fatty acid methyl esters (FAMEs) using gas chromatography (GC). Aliquots of the lipids extracted were used to prepare the FAME according to the method of AOAC (2000). In brief, the sample was mixed with 0.5 M methnolic solution (2% sodium hydroxide in methanol), heated at 85 ° C for 30 min, cooled and extracted with 2,2,4-trimethylpentane (Isooctane). Heptadecanoic acid (C17:0) was used as an internal standard. The FAME samples were injected, separated and identified on an Agilent/HP 7890 gas chromatograph (Agilent, Palo Alto, USA) with flame ionization detector (FID) equipped with an A fused silica capillary column (100 m ×0.25 mm × 0.2 μm film thickness) (Supelco, Bellefonte, PA, USA), using helium as the carrier gas at a flow rate of 1 ml/min. The analytical conditions were: injection port temperature of 250 ° C and detector temperature of 270 ° C. Retention times of FAME standards were used to identify chromatographic peaks of the samples. Fatty acid content was calculated,

based on the peak area of internal standard and expressed as mg fatty acid/100 g dry sample.

4.3.5 Peroxide value

Peroxide value (PV) of gill, skin, and muscle of 3 tropical fish and surimi samples was determined according to Richards and Hultin (2002). Samples (1 g) were homogenized in 11 ml of chloroform/methanol (2:1, v/v). Homogenates were then filtered through Whatman No. 1 filter paper. Two milliliters of 0.5% NaCl were added to 7 ml of the filtrate. The mixtures were vortexed and then was centrifuged to separate the sample into two phases. To 3 ml of lower phase, 25 µl of 30% (w/v) ammonium thiocyanate and 25 µl of 20 mM iron (II) chloride was added to the mixture. The reaction mixture was kept for 20 min at room temperature prior to reading the absorbance at 500 nm. Blanks were prepared in the same manner, except that deionized water was used instead of ferrous chloride. A standard curve was prepared using cumene hydroperoxide at concentrations ranging from 0.5–2 ppm. PV was expressed as mg cumene hydroperoxide/kg sample after blank subtraction.

4.3.6 Enzyme extraction and partial purification

Lizardfish gill were homogenized in 0.05 M phosphate buffer (pH 6.5) with 2 mM reduced L-glutathione (≥98.0%) and 0.04% Tween-20 at ratio of 1:4 (w/v) using an IKA homogenizer (IKA Works Asia, Bhd, Selangor, Malaysia). The homogenate was centrifuged at 15,000×g (Sorvall Legend MACH 1.6/R, Thermo Electron LED GmbH, Lengensellbold, Germany) for 20 min. Supernatant was collected and fractionated by 40-70% ammonium sulfate. Precipitated proteins were collected by centrifugation at 15,000× g for 30 min at 4 ° C. The precipitates were dissolved in 0.05 M phosphate buffer (pH 6.5) with 2 mM L-glutathione reduced (≥98.0%) and 0.04% Tween-20 and applied to Sephacryl-200 column equilibrated with 50 mM phosphate buffer (pH 6.5) with 2 mM reduced L-glutathione and 0.04% Tween-20 and then eluted with the same buffer. Five-ml fractions were collected at a flow rate of 1 ml/min. Fraction containing lipoxygenase activity were pooled and concentrated using 10-kDa MWCO ultrafiltration membrane (Amicon, Billerica, MA, USA). The concentrated sample was applied to

Sephacryl-200 column and eluted at a flow rate of 0.3 ml/min. The concentrated sample was applied to DEAE-Sepharose column equilibrated with 50 mM phosphate buffer, pH 6.5 and eluted using a linear gradient of 0-2.0 M NaCl, 50 mM phosphate buffer, pH 6.5. Two-ml fractions were collected at flow rate of 1 ml/min. Fractions containing lipoxygenase activity were pooled and subsequently dialyzed overnight against the 50 mM phosphate buffer, pH 6.5 with two changes of the buffer using SnakeSkinTM pleated dialysis tubing with 10 kDa molecular weight cut-off (MWCO) (Pierce Chemical Co., Rockford, IL, USA). The dialysates were concentrated using 10-kDa MWCO ultrafiltration membrane and used for LOX characterization. Protein content was carried out by the modified Bradford method using BSA as a standard (Bradford, 1976).

4.3.7 Lipoxygenase assay

LOX activity was measured by a UV spectrophotometer at 234 nm, which monitored the formation of conjugated dienes. Linoleie acid was used as a substrate and prepared according to method of Patel, Patel, and Thakkar (2015). LA of 45 mg was mixed with 90 mg of Tween-20 in 1 ml of DI water and emulsifying in 150 µl of 1 N NaOH. Final volume was brought to 20 ml with 0.05 M phosphate buffer (pH 6.5). The reaction mixture was prepared by adding 1.3 ml 50 mM phosphate buffer, pH 6.5, containing 1 mM glutathione and 0.04% Tween-20 and 100 µl crude extract. The reaction mixture was initiated by adding 100 µl of substrate. Absorbance at 234 nm of the mixture were recorded in 3 min. One unit of LOX activity [U] was defined as an increase in absorbance of 0.001 at 234 nm in 1 min under the specified condition.

4.3.8 Biochemical characterization

4.3.8.1. Temperature and pH optima

The effect of temperature on the partially-purified LOX activity was investigated in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.01%Tween-20 using LA as a substrate at 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 ° C, for 5 min. The optimum pH was investigated at 25 ° C at various pH levels of pH 3, 4,

5, 5.5 using 50 mM sodium acetate; pH 5.5, 6.0, 6.5, 7, 7.5, 8 using 50 mM phosphate buffer; pH 8, 8.5, 9 using 50 mM Tris-HCl; and pH 9, 10, 11 using 50 mM borate buffer.

4.3.8.2 pH stability

pH stability was determined by pre-incubating the partially purified LOX at various pH levels including pH 3 -6 in sodium acetate, pH 6 -7.5 in phosphate buffer, 9.5 in Tris-HCl, at final concentration of 50 mM. The mixture was pre-incubated at 25 ° C for 30 min. When preincubation time was reached, LOX activity was determined. Control samples at each pH value were run in the same manner except that their activities were determined immediately without preincubation.

4.3.8.3 Thermal stability.

Thermal stability of the partially-purified LOXwas determined by preincubating the enzyme at various temperatures (10 - 80 ° C) for 30 min. When preincubation time was attained, samples were rapidly cooled in ice water for 20 min. Some precipitates were noticed at high temperatures which was removed by centrifugation. Lipoxygenase activity was determined as described above. Activities of the control sampleswithout preincubation were determined at 25 ° C and pH 7.5.

4.3.8.4 Substrate specificity.

Substrate specificity of the partially-purified LOX was determined using various substrates, including oleic acid, linoleic acid, linolenic acid, arachidonic acid, EPA, and DHA at 2.5 mM. LOX activity was determined as described above.

4.3.8.5 Effect of inhibitors and ions on activity.

The effect of various inhibitors on LOX activity was determined using various substances, namely, nordihydroguaiaretic acid (NDGA), ethylenediaminetetraacetic acid (EDTA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid, and N-propyl gallate, at final concentration of 1 mM. The effect of metal ions ($CuCl_2$, $FeCl_3$, $MnCl_2$, $HgCl_2$, and $ZnSO_4$) and mono- and divalent cations (NaCl, KCl, $MgCl_2$, and $CaCl_2$) at 1 mM was also investigated.

4.3.9 Statistical analyses

All experiments were conducted in triplicate, and results were analyzed by one-way analysis of variance (ANOVA). Significant differences in mean values were analyzed by Duncan's multiple range mean comparison test within the 95% confidence interval using. All data were analyses with the SPSS 17.0 statistical package (SPSS Ltd., Working, Surrey, UK).

4.4 Results and discussion

4.4.1 Lipid composition in various tissues

Total lipid contents and amounts of each fatty acid of gill, skin, and muscle of 3 tropical fish are listed in Table 4.1. Gill of lizardfish contained the highest total lipid content, which was about 5 and 29 times higher than that of goatfish and threadfin bream, respectively. Total lipid contents of skin and muscle of all 3 species ranged 20.6-37.0 g/kg dry weight. All tissues of tropical fish showed higher saturated fatty acid (∑SFA) content than polyunsaturated fatty acid (∑PUFA) and monounsaturated fatty acid (∑MUFA), while gill contained the highest of ∑SFA, ∑MUFA, and ∑PUFA. Wu, Forghani, Abdollahi, and Undeland (2022) reported that ∑SFA and ∑MUFA of head were higher than fillet, whereas ∑PUFA was the highest in fillet. Twenty-two marine fish species from the Pearl River Estuary contain high contents of SFA and low contents of PUFA, which might be associated with their differed dietary composition (Zhang et al., 2020). Among 3 species, all tissues of lizardfish showed the lowest ∑SFA and ∑PUFA, whereas all tissues of threadfin bream exhibited the highest ∑MUFA. Predominant SFA and MUFA in all tissues of all species was palmitic and oleic acid, respectively, whereas DHA and EPA are the main PUFA. Goncalves et al. 2021 reported that PUFAs of muscle were major fatty acids in twelve of fourteen marine fish species from Northeast coast of Brazil and EPA and DHA were major fatty acids in most species studied. n-3 PUFAs in marine fish are mostly found in phospholipids (Burri, Hoem, Banni, and Berge, 2012). Our results indicated that amount of total lipid and fatty acid contents of 3 tropical species differed among speices and tissues.

Table 4.1 Total extracted lipids and amount of each fatty acid of various tissues of 3 tropical species used in surimi production.

	Gill			Skin			Muscle		
	Goatfish	**Lizard**	**Threadfin beam**	**Goatfish**	**Lizard**	**Threadfin beam**	**Goatfish**	**Lizardfish**	**Threadfin bream**
Total lipid (g/kg dry weight)	30.80 ± 1.80c	162.34 ± 7.04d	5.66 ± 0.32a	22.77 ± 0.32b	22.52 ± 0.24b	37.04 ± 4.40c	26.41 ± 0.50b	23.53 ± 0.58b	20.67 ± 0.18b
FA content (mg/100 g dry weight)									
C12 : 0	8.75 ± 0.38	1.94 ± 0.22	9.77 ± 0.73	4.58 ± 0.50	1.12 ± 0.11	6.51 ± 0.31	1.75± 0.12	ND	1.85 ± 0.16
C13 : 0	4.66 ± 0.18	0.81 ± 0.09	3.40 ± 0.37	3.09 ± 0.48	0.48 ± 0.00	2.31 ± 0.05	1.06 ± 0.21	ND	0.78 ± 0.07
C14 : 0	222.15 ± 9.17	84.74 ± 6.90	177.27 ± 16.72	129.47 ± 16.04	65.73 ± 0.18	116.34 ± 14.44	45.59 ± 6.17	19.37 ± 1.91	36.63 ± 3.01
C15 : 0	ND	20.03 ± 1.61	50.92 ± 0.46	36.65 ± 4.05	12.55 ± 0.45	37.79 ± 0.46	15.23 ± 0.02	6.34 ± 0.63	12.85 ± 0.04
C16 : 0 (Palmitic acid)	1052.97 ± 41.24	485.86 ± 39.65	1244.95 ± 23.62	529.67 ± 18.21	327.12± 1.62	877.67 ± 108.41	332.28 ± 1.55	230.01 ± 22.72	406.47 ± 17.18
C17 : 0	410.08 ± 10.44	187.56 ± 12.50	432.75 ± 11.68	217.33 ± 8.99	117.75 ± 2.60	245.83 ± 28.89	140.14 ± 8.50	106.54 ± 10.52	136.14 ± 3.00
C18 : 0	516.79 ± 20.16	196.04 ± 20.08	611.57 ± 19.39	286.76 ± 8.93	116.35 ± 2.04	393.63 ± 45.34	162.62 ± 1.80	81.61 ± 8.06	171.02 ± 10.66
C22 : 0	6.84 ± 0.32	3.65 ± 0.41	9.16 ± 0.45	4.36 ± 0.13	2.78 ± 0.03	7.09 ± 0.36	2.57 ± 0.08	1.81 ± 0.18	3.94 ± 0.37
C23 : 0	ND	ND	0.05 ± 0.00	2.70 ± 0.44	4.18 ± 0.03	8.24 ± 0.85	1.97 ± 0.13	0.90 ± 0.09	2.15 ± 0.00
∑SFA	2222.23 ± 81.89b	980.61 ± 81.46a	2545.48 ± 73.69b	1214.62 ± 57.78ab	648.06 ± 7.06a	1695.40 ± 199.11b	705.21 ± 19.82b	446.58 ± 44.11a	771.82 ± 34.48b
C14 : 1	67.98 ± 2.70	ND	2.52 ± 0.37	0.63 ± 0.11	0.63 ± 0.00	1.83 ± 0.23	ND	ND	ND
C16 : 1	260.73± 10.69	102.09 ± 8.38	259.53 ± 31.37	133.81 ± 13.72	77.57 ± 0.14	195.14 ± 21.56	48.28 ± 6.17	32.70 ± 3.23	67.48 ± 3.21
C17 : 1	14.20 ± 0.59	ND	ND	7.23 ± 1.04	4.72 ± 0.04	9.21 ± 0.36	3.28 ± 0.75	ND	2.55 ± 0.05
C18 : 1n9t (Oleic acid)	361.95 ± 15.64	146.10 ± 13.24	554.58 ± 24.68	180.24 ± 13.52	89.27 ± 0.62	391.34 ± 41.93	80.95 ± 4.02	61.26 ± 6.05	170.05 ± 3.53
C18 : 1n9c	147.90 ± 6.16	65.94 ± 5.42	184.18 ± 15.66	73.57 ± 3.49	38.13 ± 0.10	120.45 ± 15.16	35.94 ± 1.86	25.61 ± 2.53	46.33 ± 0.30
C20 : 1	22.00 ± 1.00	6.49 ± 1.39	24.13 ± 0.70	5.99 ± 0.31	4.76 ± 0.07	10.44 ± 4.60	2.63 ± 0.21	1.77 ± 0.18	2.34 ± 0.10
C22 : 1n9	8.62 ± 0.37	ND	ND	ND	ND	ND	105.71 ± 6.92	ND	126.56 ± 13.38
C24 : 1	23.49 ± 14.83	2.69 ± 0.60	6.76 ± 0.31	2.30 ± 0.48	9.28 ± 0.28	46.90 ± 1.54	17.88 ± 0.17	5.37 ± 0.53	26.97 ± 3.67
∑MUFA	906.86 ± 51.98d	323.48 ± 29.28b	1031.71 ± 73.10d	403.76 ± 32.67c	224.38 ± 1.25b	775.31 ± 85.25cd	294.67 ± 20.10b	126.71 ± 12.52a	442.28 ± 24.23c
C18 : 2n6c (Linoleic acid)	64.81 ± 2.57	24.66 ± 2.73	53.23 ± 3.75	43.34 ± 3.83	17.52 ± 0.21	4.22 ± 0.39	22.54 ± 0.94	9.84 ± 0.97	18.99 ± 0.02
C18 : 3n6	6.71 ± 0.27	2.73 ± 0.25	4.89 ± 0.63	3.93 ± 0.28	2.34 ± 0.07	3.77 ± 0.16	1.79 ± 0.11	0.95 ± 0.09	1.74 ± 0.33
C18 : 3n3	13.16 ± 0.50	3.52 ± 0.35	15.62 ± 0.26	8.06 ± 0.63	2.50 ± 0.07	9.00 ± 0.92	2.91 ± 0.43	1.10 ± 0.11	2.73 ± 0.14
C20 : 2	18.94 ± 0.84	5.45 ± 0.48	22.49 ± 1.32	13.18 ± 1.14	3.49 ± 0.12	13.62 ± 0.91	6.18 ± 0.38	2.34 ± 0.23	5.79 ± 0.15
C20: 3n3	4.77 ± 0.21	4.04 ± 0.48	10.61 ± 1.37	4.85 ± 0.29	2.84 ± 0.06	6.30 ± 0.72	ND	48.33 ± 4.77	ND
C20 : 4n6 (Arachidonic acid)	183.51 ± 7.10	116.83 ± 8.72	244.31 ± 2.39	104.12 ± 3.41	67.47 ± 1.52	150.76 ± 16.15	2.43 ± 0.20	2.37 ± 0.23	3.97 ± 0.09
C22 : 2n2	6.43 ± 0.19	2.27± 0.81	0.05 ± 0.00	3.33 ± 0.16	1.12 ± 0.10	4.30 ± 0.06	ND	ND	ND
C20 : 5n3 (EPA)	291.45 ± 10.87	129.07 ± 13.42	290.29 ± 33.70	124.38 ± 4.72	96.97 ± 3.78	227.54 ± 34.53	92.35 ± 1.96	76.31 ± 7.54	107.56 ± 1.42
C22 : 6n3 (DHA)	877.34 ± 34.73	465.92 ± 37.05	705.48 ± 53.71	604.39 ± 20.19	393.35 ± 11.89	523.97 ± 95.06	570.04 ± 21.44	356.24 ± 35.19	374.61 ± 55.10
∑PUFA	1467.12 ± 57.29d	754.50 ± 64.29b	1353.96 ± 97.51d	909.58 ± 34.65c	587.60 ± 17.80a	943.48 ± 148.91c	698.25 ± 25.46b	497.49 ± 49.14a	515.39 ± 57.37a

*SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid

[a-d] Mean values in the same column with different superscripts differ significantly ($P < 0.05$)

4.4.2 LOX activity

LOX activity and peroxide values of gill, skin, and muscle of tropical fish namely goatfish, lizardfish, and threadfin bream are listed in Table 4.2. LOX activities varied with tissues. Gill of lizardfish exhibited the highest LOX activity, which was about 2 times higher than threadfin bream muscle ($p < 0.05$). Gill of Baltic herring showed the lowest activity, which was comparable to muscle and skin tissue. (Stodolnik and Samson, 2000). Muscle of lake herring showed higher LOX activity than skin tissue (Wang, Miller, and Addis, 1991). Hsieh, German, and Kinsella (1988) reported that gill and skin tissues of freshwater fish species contained active LOX activity and there was no detectable LOX activity in muscle. Wu et al. (2022) reported that LOX activity of head was higher than fillet in sorted herring and this enzyme had a significant impact on its lipid oxidation.

PVs of gill tissue of all 3 species were the highest compared to other tissues with threadfin bream being the highest ($p < 0.05$). Lipid oxidation occurred during postmortem handlings and was influenced by tissue type, and age. Fresh fish is known to contain peroxide which is naturally produced and can react with biological molecules. Fu et al. (2009) reported LOX from silver carp muscle caused a faster initial reaction of lipid oxidation than hemoglobin but had lower lipid hydroperoxide value and severe fishy odor. Our results indicated that LOX activity was not related to PV.

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Table 4.2 LOX activity and peroxide value of various tissues of 3 tropical species used in surimi production.

Tissues	species	LOX activity (U/mg protein)	Peroxide value (mg hydroperoxide/kg dry weight)
Gill			
	Goatfish	48.93 ± 0.87b	67.43 ± 2.54c
	Lizardfish	376.56 ± 22.0d	66.79 ± 3.94c
	Threadfin Bream	55.66 ± 0.32b	78.23 ± 4.87d
Skin			
	Goatfish	26.19 ± 1.57a	37.56 ± 2.4a
	Lizardfish	50.67 ± 1.08b	41.58 ± 3.52a
	Threadfin Bream	60.67 ± 0.18b	51.77 ± 3.22b
Muscle			
	Goatfish	30.53 ± 0.54a	32.54 ± 3.45a
	Lizardfish	75.62 ± 0.08b	42.57 ± 1.33a
	Threadfin Bream	137.04 ± 4.4c	48.11 ± 4.51b

[a-d] Mean values in the same column with different superscripts differ significantly ($P < 0.05$)

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4.4.3 Changes of LOX activity during lizardfish surimi processing

The changes in LOX activities (units/g sample) during lizardfish surimi processing are shown in Figure 4.1. A significant decrease in LOX activities was detected throughout surimi processing ($p < 0.05$). A decrease in activity suggested that the enzyme can be removed by washing. There was approximately 57.5% reduction of lipoxygenase activity of lizardfish mince after washing. Washing not only removes fat and undesirable substances such as blood, pigments and odorous contents, but also increases insoluble proteins, including myofibrillar protein, connective tissue proteins, as well as membrane-bound cholesterol and lipids. Yamashita, Nakamura, Noguchi, Niki, and Kühn, (1999) reported LOX are capable of specially oxidizing phospholipids and cholesterol esters in membranes and lipoproteins. 15-LOX was localized in the cytosol but also bound to intracellular membranes (Brinckmann et al., 1988). Mince reached it minimum level of LOX activities by a dewatering with a 70% decrease. However, the enzyme residual activity in surimi remains after frozen storage (57.85 U/g sample). LOX activity has been reported in silver carp surimi products at setting stage LOX activity reached 4.19-4.81 U/min·g (An et al., 2022). The enzyme residual activity remaining in the surimi could partly contribute to lipid oxidation of surimi during frozen storage.

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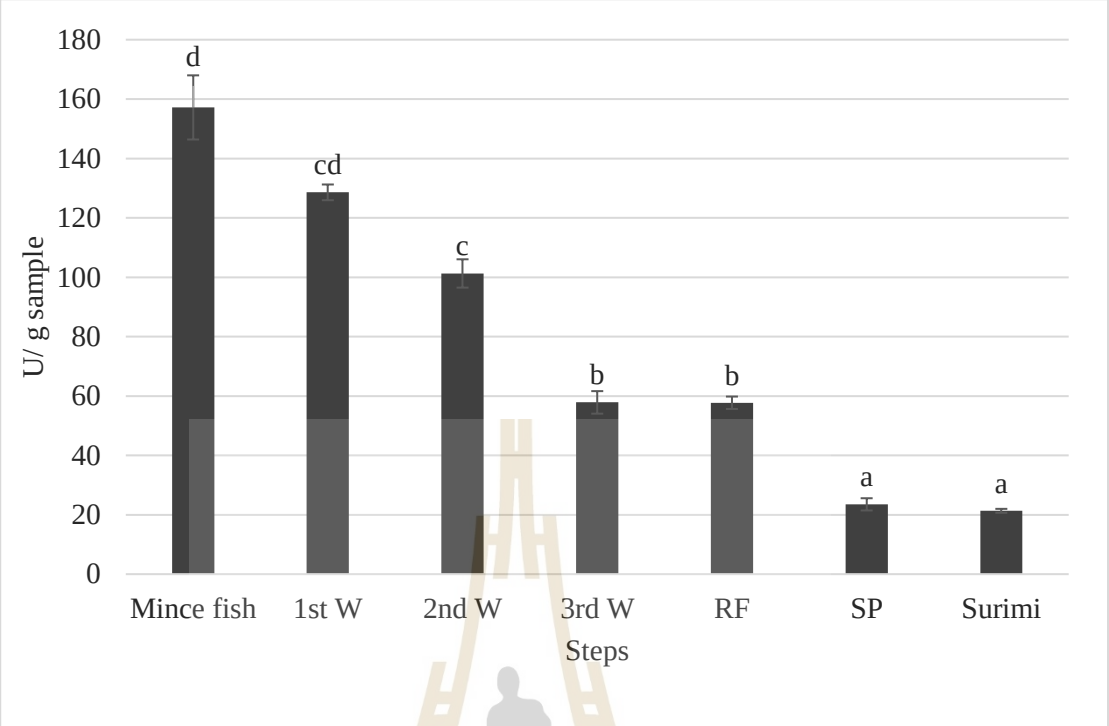

Fig. 4.1 Changes of LOX activity during lizardfish surimi processing (1st W = First washing, 2nd W = Second washing, 3rd W = Third washing, RF = Refining, SP = Screw pressing)

4.4.4 Changes of fatty acid during surimi processing

Total extracted lipids and fatty acid contents of lizardfish surimi processing is shown in Table 4.3. A decrease in total lipids was detected in mince from screw pressing and lizardfish surimi (LZ surimi) ($p<0.05$). Total lipids of mince from screw pressing and LZ surimi decreased by 45.81% and 56.7% as compared to minced fish. Proportions of fatty acid were similar in minced fish and in LZ surimi. Amount of monounsaturated fatty acid (∑MUFA) lower than saturated fatty acid (∑SFA) and polyunsaturated fatty acid (∑PUFA). A decrease in fatty acid contents were detected in screw pressing step and surimi ($p<0.05$). ∑SFA, ∑MUFA, and ∑PUFA decreased by 63.67%, 65.76%, and 62.79% at surimi step as compared to minced fish. A decrease in total lipids and fatty acid contents suggested that the lipid can be removed by dewatering by screw press. Surimi has a higher phospholipid content than minced could be explained by the membrane polar lipids, such as phospholipids, interacting

with proteins and consequently being less easily removed than neutral lipids (Eymard et al., 2005). These phospholipids are highly unsaturated and often in contact with muscle heme iron and very sensitive to oxidation. According to Clark et al. (2011) LOX may act on phospholipids in which fatty acids are esterified to the glycerol backbone. Omega-3 PUFA in marine fish are mostly found in phospholipids form (Burri et al., 2012). In our study, palmitic acid as the predominant SFA, oleic acid is highest MUFA, whereas DHA and EPA are the main PUFA in LZ surimi. These results indicating that PUFA in surimi can serve as a substrate of LOX. Eymard et al. (2005) reported that the last dewatering stage of mackerel surimi production can remove a major portion of lipid oxidation products. However, our findings indicated that PUFA and LOX were still present in surimi, which both substrate and enzyme could lead to oxidation of lipids during storage, which has not been reported.

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Table 4.3 Total extracted lipids and amount of each fatty acid of the samples taken during surimi manufacturing.

	Mince fish	Mince from refining	Mince from screw pressing	Surimi
Total lipid (g/ kg dry sample)	18.71 ± 3.62b	17.94 ± 2.25b	10.14 ± 1.07a	8.22 ± 1.45a
FA content (mg/ 100 g dry sample)				
C14 : 0	42.33 ± 6.38	32.96 ± 32.96	19.89 ± 3.64	13.91 ± 0.80
C15 : 0	15.19 ± 2.30	12.25 ± 1.60	7.07 ± 1.26	5.16 ± 0.29
C16 : 0	351.74 ± 46.48	303.7 ± 40.69	162.27 ± 27.51	127.87 ± 6.99
C17 : 0	159.18 ± 13.23	153.55 ± 15.51	75.05 ± 7.14	62.2 ± 1.14
C18 : 0	137.65 ± 18.72	111.58 ± 15.15	61.75 ± 10.81	47.52 ± 2.63
C20 : 0	1.70 ± 0.16	1.37 ± 0.17	0.90 ± 0.05	0.53 ± 0.05
C21 : 0	1.64 ± 0.24	1.24 ± 0.16	0.77 ± 0.15	0.50 ± 0.06
C23 : 0	58.18 ± 7.14	49.25 ± 6.56	26.26 ± 4.36	20.54 ± 1.07
∑SFA	767.60 ± 94.7b	665.95 ± 84.5b	353.95 ± 54.92a	278.23 ± 13.03a
C16 : 1	57.67 ± 8.31	47.00 ± 644	27.36 ± 4.69	19.63 ± 1.11
C18 : 1n9t	0.51 ± 0.09	0.45 ± 0.12	0.23 ± 0.04	0.08 ± 0.00
C18 : 1n9c	106.3 13.51	87.55 ± 11.75	46.34 ± 8.00	36.95 ± 2.14
C20 : 1	6.00 ± 1.50	4.28 ± 0.59	2.51 ± 0.45	2.20 ± 0.04
C22 : 1n9	2.35 ± 0.33	1.81 ± 0.26	1.04 ± 0.18	0.88 ± 0.27
C24 : 1	1.40 ± 0.13	0.92 ± 0.11	0.60 ± 0.08	0.35 ± 0.02
∑MUFA	174.24 ± 23.87b	142.01 ± 10.27b	78.07 ± 13.45a	60.08 ± 3.65a
C18 : 3n6	1.77 ± 0.27	1.41 ± 0.19	0.81 ± 0.15	0.58 ± 0.09
C18 : 3n3	4.83 ± 3.97	1.87 ± 0.22	1.04 ± 0.18	2.84 ± 0.18
C20 : 2	4.45 ± 0.59	3.71 ± 0.49	1.99 ± 0.34	1.55 ± 0.08
C20 : 3n6	3.11 ± 0.39	2.71 ± 0.36	1.42 ± 0.23	1.14 ± 0.05
C20: 3n3	2.15 ± 0.35	1.6 ± 0.23	0.97 ± 0.19	0.27 ± 0.02
C20 : 4n6	85.9 ± 10.31	79.03 ± 10.73	38.87 ± 6.17	32.61 ± 1.68
C22 : 6n3	629.26 ± 71.65	599.18 ± 80.92	295.61 ± 46.61	233.42 ± 10.85
∑PUFA	731.50 ± 87.53b	689.52 ± 93.14b	340.71 ± 53.87a	272.41 ± 13.21a

[a] Different letter in the same column indicate difference at $P < 0.05$.

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4.4.5 Partial purification of LOX from Lizardfish Gill.

Based on purification scheme, ammonium sulfate precipitation of 40-70% effectively removed contaminant proteins, rendering an increase of 4.55 folds of purity, and about 118% activity when compared to the crude extract (Table4.4). Wang et al. 2012 reported 40% saturated ammonium sulfate was effective for LOX fractionation in grass carp muscle. A Sephacryl S-200 column based on size exclusion chromatography was used in the second purification step. The recovery of LOX activity was 12.89 % with 12.01-fold purification (Figure 4.1). The active fraction from the first gel filtration was collected, concentrated, pooled and loaded again onto the Sephacryl S-200 column with a lower flow rate. Two protein peaks were well separated, and LOX activity was found in the minor protein peak. LOX recovery after the second gel filtration was 8.46 % with 15.85-fold purification (Table 4.4). Fractions giving LOX activity were pooled and loaded into a DEAE-Sepharose ion-exchange column. Thirty-five fractions were obtained from gradient elution and 5 fractions eluted at 0.8 M NaCl showing LOX activity (Fig. 4.3). An overall 22.43-fold purification was achieved with a yield of 3.52 %. The specific activity of pure LOX reached 1267.64 units mg^{-1} when linoleic acid was used as a substrate. LOXs have a single atom of non-heme iron essential for activity (Newcomer and Brash, 2015).

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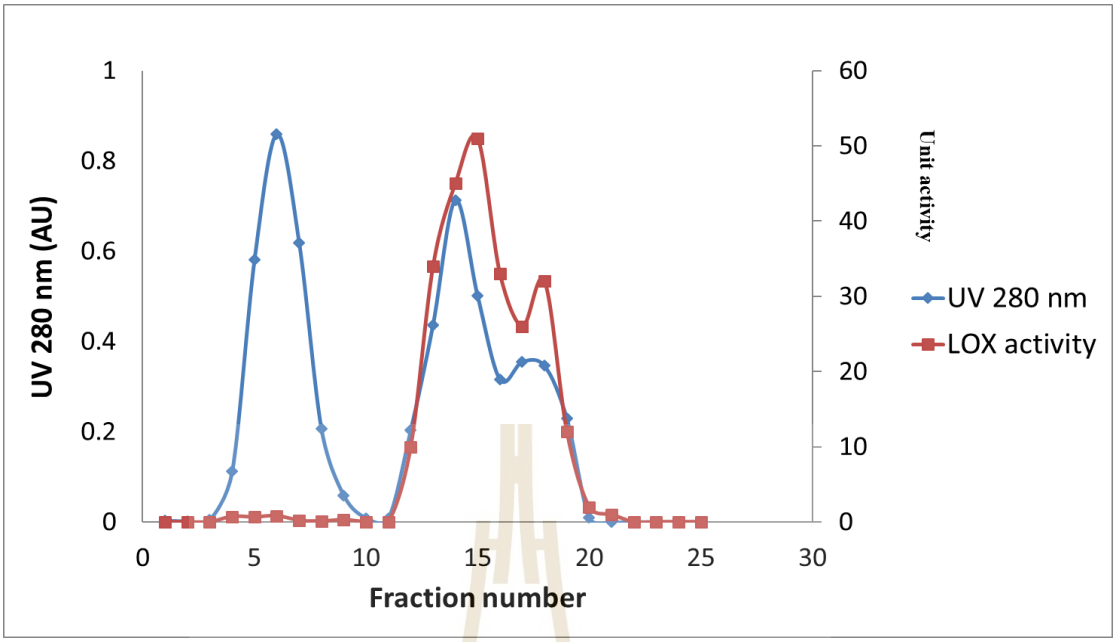

Fig. 4.2 Elution profile of lipoxygenase from lizardfish gill on a Sephacryl S-200 column. Fractions of 5 ml were collected at flow rate of 1 ml/min.

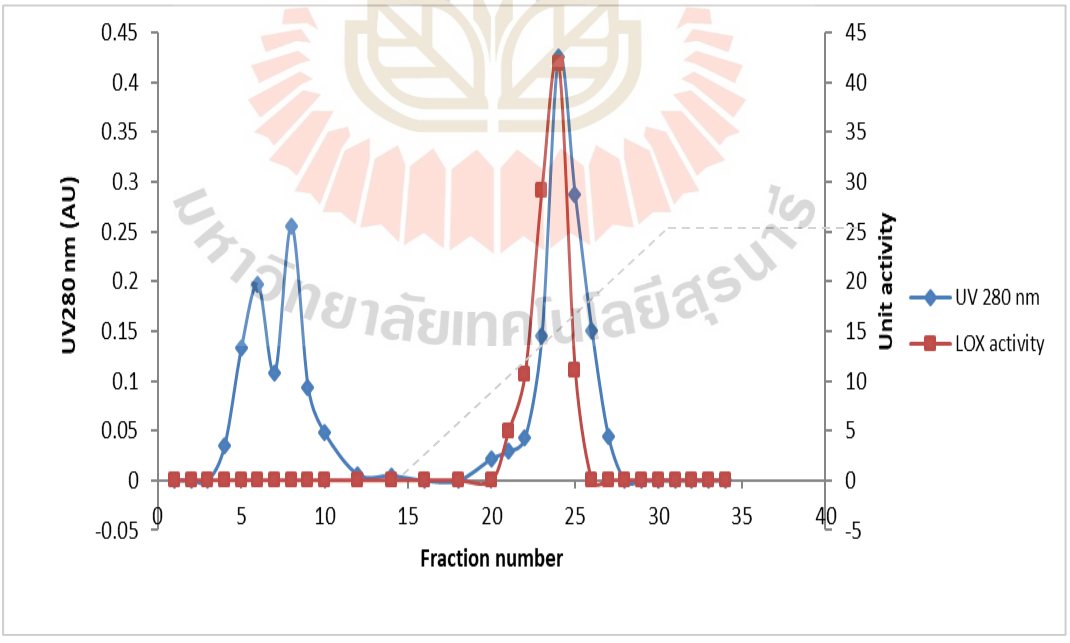

Fig. 4.3 Elution profile of lipoxygenase from lizardfish gill on a DEAE-Sepharose ion-exchange column.

Table 4.4 Purification table of lizardfish gill lipoxygenase

Step	Total protein (mg)	Total activity (Unit)*	Specific activity (Unit/mg)	Purity (fold)	Yield (%)
Crude extract	229.50	12973.21	56.53	1.00	100.00
$(NH_4)_2SO_4$ precipitation	59.49	15314.29	257.42	4.55	118.05
Sephacryl S-200 (Flow rate 1 ml/min)	2.46	1671.79	679.07	12.01	12.89
Sephacryl S-200 (Flow rate 0.3 ml/min)	1.22	1097.14	896.13	15.85	8.46
DEAE-Sepharose	0.36	456.35	1267.64	22.43	3.52

*One unit of enzyme activity is defined as an increase in absorbance at 234 nm of 0.001 after 1 minute under the specified condition.

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4.4.6 Optimum temperature and pH.

Optimum temperature of the partially-purified LOX was at 25 ° C (Figure 4.4A). It showed high activity (82-98%) over a wide temperature range of 15-35 ° C. High activity of fish LOX at low temperatures suggests that it may contribute to initiation of fish lipid oxidation at refrigerated conditions. At 40 ° C, 60% of activity was retained. However, only minimal activity was observed at temperatures above 45 ° C. This was quite different from LOX purified from cold water species fish including mackerel and trout, which typically showed activity only at temperature of 20-25 ° (Hong et al., 1994; Hsieh et al.,1988). Enzymes from fish warm water habitat exhibited higher optimal temperature, indicating that it might be effect flavor quality much higher than cold water species.

Lizardfish had optimum activity around pH 7.5 with the activity rapidly declining below pH 6.0 and above pH 8.0 (Figure 4.4B). The enzyme was less sensitive to pH change in the acidic range than in the alkaline range. A decrease in pH from 7.5 to 6.0 resulted in the loss of 20% of the activity, while raising pH from 7.5 to 9.0 reduced the activity by 80%. A similar optimum pH was observed among LOX in teleost fishes and rainbow trout (German and Creveling,1990; Hsieh et al.,1988). LOX from mackerel muscle and gill showed optimum pH at 5.6 and 4.5 respectively (Banerjee et al., 2002; Hong et al., 1994), which was lower than the value observed in this study. The LOX distributed in gill and ovary of grey mullet showed optimum activity at pH 8 and pH 6.5, respectively (Cadwallader, 2000).

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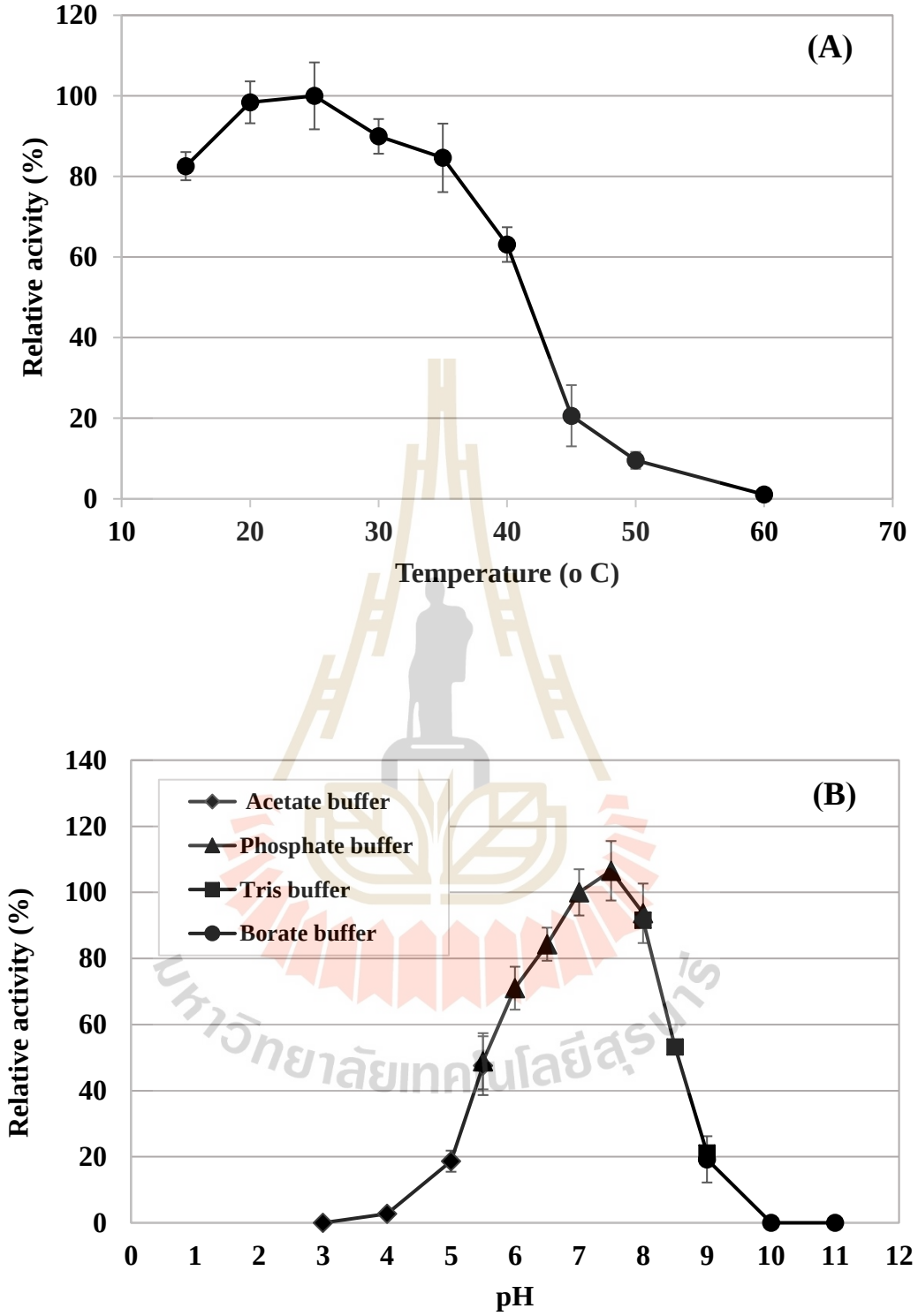

Fig. 4.4 Temperature (A) and pH profile (B) of partially-purified lipoxygenase from lizardfish gill.

4.4.7 Thermal stability and pH stability

The partially-purified lipoxygenase showed high stability at 10-50 ° C, and its stability rapidly decreased at temperature 60 ° C with 60% of the activity remaining (Figure 4.5A). Heating to 70 ° C and 80 ° C completely inactivated the enzyme. A decrease in activity is probably due to irreversible changes in the tertiary structure of the enzyme. The activity of LOX from mackerel muscle and trout decreased to 20% of the original activity, after incubation at 40 ° C for 10 min and over 80 % after incubation at 50 and 70 ° C for 10 min, respectively (Banerjee et al., 2002; Hsieh et al.,1988). An et al. (2022) reported LOX activity was high in the silver carp surimi gel at 40 ° C, and rapidly decreased at temperature 90 ° C for 10 min. Tolasa Yılmaz, Çaklı, Şen Yılmaz, Kırlangıç, and Lee (2018) found that increasing temperature (from 0 to 10 ° C) increased LOX activity significantly in fresh and frozen sardine fillets and mince. LOX activity in pork were completely inactivated at 50 ° C with high pressure 600 MPa (Huang, Wang, Wu, and Li, 2016). The instability of LOX above 50 ° C may provide an approach for controlling enzyme activity and help improve flavor quality of lizardfish.

The partially purified lipoxygenase was highly stable at a pH range of 6-8 (Figure 4.5B). The LOX from mackerel muscle and sardine were stable over broad pH ranges of 4-11 and 6-9, respectively (Banerjee et al., 2002; Mohri et al., 1992). The pH value of 6-8 falls in the postmortem pH of lizardfish, suggesting that enzyme likely remain active at postmortem during ice or refrigerated storage. Thiansilakul, Benjakul, and Richards, (2011) reported the highest lipid oxidation and off-odor were observed in washed Asian seabass mince at pH 6.0. Thus, lipid oxidation catalyzed by LOX likely occur at postharvest storage, which might be a main reason of distinct flavor of lizardfish meat.

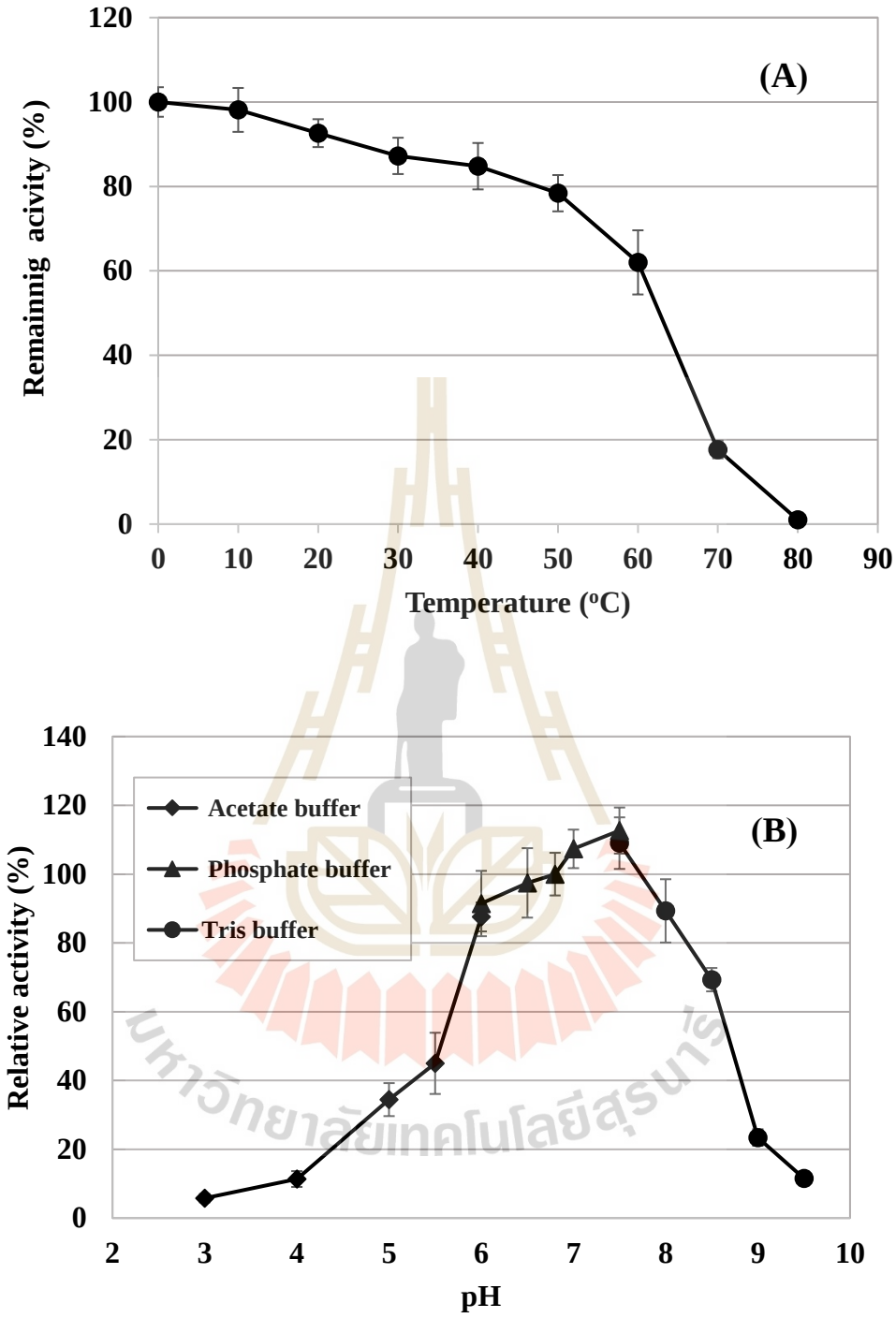

Fig. 4.5 Thermal (A) and pH stability (B) of the partially-purified lipoxygenase. Thermal stability was carried out by preincubating the enzyme at various temperature at pH 7.5 for 30 min, and pH stability was determined by preincubating the enzyme at varying pH values and determined activity at 25 °C for 30 min.

4.4.8 Substrate specificity

The activity of lipoxygenase toward different fatty acids is shown in Figure 4.6. The highest activity was observed with eicosapentaenoic acid (EPA), followed by linoleic acid and docosahexaenoic acid (DHA). Substrate specificities of the enzyme appear to vary with sources of LOX. LOX from trout and mackerel efficiently oxidized n-3 fatty acids such as DHA and EPA (Hsieh et al., 1988; Harris and Tall 1994). LOX from carp gill preferred arachidonic acid (Iijima et al., 2000), while sardine skin LOX showed maximal activity toward linoleic acid (Mohri et al., 1990). Gill LOX of rainbow trout exhibited reactivity toward arachidonic, EPA, and DHA, but low reactivity toward linoleic acid (German and Creveling, 1990). Banerjee et al. (2002) reported that the highest activity of mackerel muscle was observed when linoleic acid was used as a substrate, followed by linolenic acid, DHA and arachidonic acid. Oleic acid with a single double bond is not a substrate for LOX, resulting in relatively low activity (Figure 4.5). Therefore, high unsaturation may enhance the activity of lizardfish LOX. In this study, related to Table 4.1. Gill, skin, and muscle of lizardfish are rich in DHA, EPA, and linoleic acid, which can serve as substrates for LOX.

4.4.9 Inhibitors and Metal Ions.

Ethylene diamine tetra-acetic acid (EDTA) at 1 mM completely inhibited LOX activity based on both substrates of LA and EPA (Table 4.5). LOX inhibition was also observed in the presence of BHA, BHT, ascorbic acid and N-propyl gallate. EDTA inhibits the enzyme by chelating iron. LOXs have a two-domain structure of β-barrel domain and α-helical catalytic domain containing a single atom of non-heme iron essential for activity. Moreover, Wang et al. (2019) found that EDTA can cause the content of α-helix and β-sheet inside the porcine 12-LOX molecule to decreased and result in LOX activity change. LOX catalyzes the peroxidation of cis, cis-1,4-pentadiene structures. In its oxidized state, LOX ($Fe^{3+)}$ can abstract a proton from a PUFA and reduce itself to LOX (Fe^{2+}), producing a fatty acid free radical. Antioxidants such as BHA, BHT, propyl gallate, and ascorbic acid can effectively scavenge free radicals by donating labile hydrogen to oxygen radicals derived from fatty acids. (Festjens et al., 2006). Propyl gallate inhibited LOX activity in soy milk more than BHA, BHT, and ascorbic acid (Vijayvaragiya and Pai, 1991).

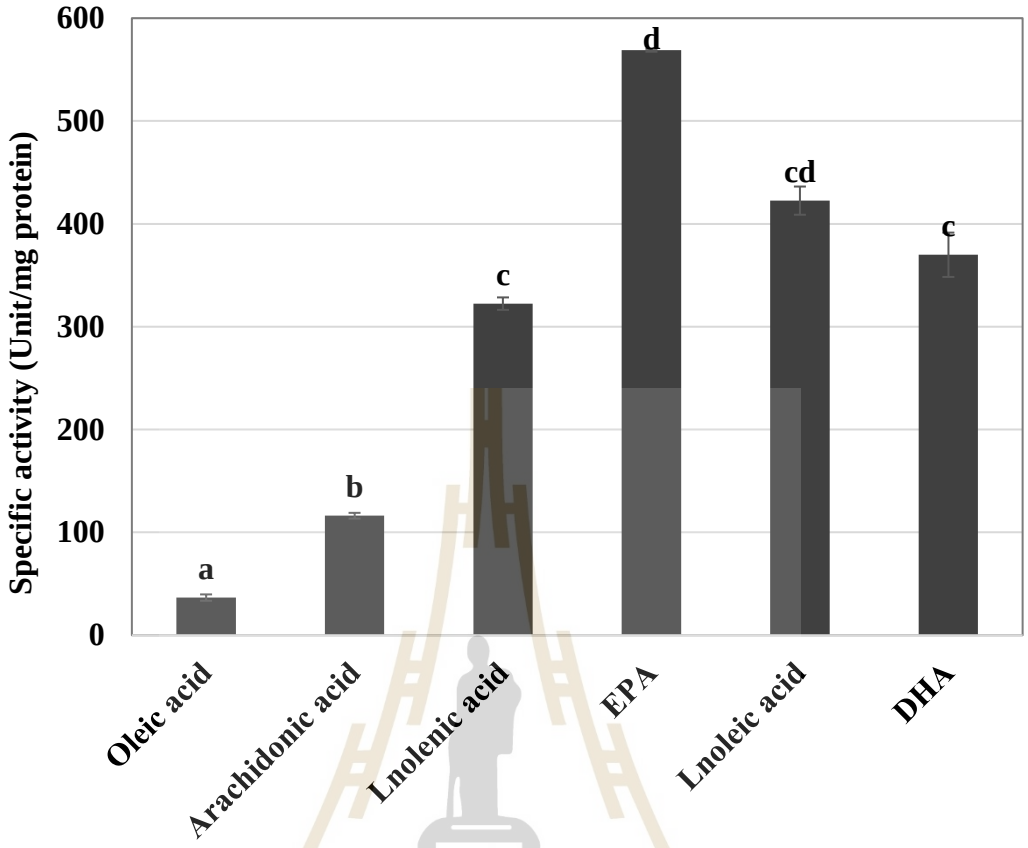

Fig. 4.6 Effect of different fatty acids on LOX activity (EPA = Eicosapentaenoic acid, DHA = Docosahexaenoic acid)

Metal ions play an important role in the activity of fish lipoxygenases, acting as activators of an enzyme through its binding with a substrate or release of its active center. The enzyme activity was significantly increased by Fe^{2+}, Na^{+} and Ca^{2+} for both substrates but decreased in the presence of Cu^{2+} (Table 4.4). Fe^{2+} caused an increase in LOX activity, which was clearly due to Fe^{2+} catalyzed auto-oxidation. NaCl is an effective catalyzer of LOX in the muscle tissue and roe of Baltic herring (Samson and Stodolnik, 2001). Zhang et al. (2019) reported LOX activity increased at salting and resting stages in boneless dry-cured hams during processing might be attributed to the activation of salt. However, Activity of LOX from Mackerel gill was not affected by Na^{+} (Hong et al., 1994). The type of metal ion contained in food may have a major impact on the enzyme's activities.

Table 4.5 Effects of various chemicals on the activity of lizardfish LOX toward different

Substances (1.0 mM)	Residual activity (%)	
	Linoleic acid	EPA
Control	100	100
EDTA	0	0
BHA	95	0
BHT	85	0
Ascorbic acid	60	0
N-propyl gallate	77	0
Cu^{2+}	71	68
Fe^{2+}	120	138
Mn^{2+}	98	89
Hg^{2+}	89	92
Zn^{2+}	100	98
Na^{+}	137	128
K^{+}	106	109
Mg^{2+}	99	100
Ca^{2+}	118	133

4.5 Conclusion

In the manufacturing of tropical surimi, goatfish, lizardfish, and threadfin bream are the major raw materials. It contained a high concentration of PUFA susceptible to lipid oxidation, especially that catalyzed by LOX. DHA and EPA are the two most abundant polyunsaturated fatty acids (PUFA) in tropical fishes. Lizardfish gill exhibited the highest LOX activity as compared to gill, skin, and muscle of other tropical fish. Optimal activity of lizardfish gill LOX was at 25 ° C and pH 7.5. The enzyme was stable at temperatures below 50 ° C. Lizardfish gill LOX exhibited substrate specificity toward EPA. The enzyme was completely inhibited by EDTA but activated by metal ions. Lizardfish surimi showed highly unsaturated fatty acid contents and it can serve as a substrate of LOX. The enzyme residual activity remains after washing (57.85 U/g

sample). This study suggested that LOX activity could be one of intrinsic factors affecting lipid oxidation of surimi during frozen storage.

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CHAPTER V
SUMMARY

It has been demonstrated that lipid oxidation of tilapia differed among body parts. During prolonged ice storage, the gill of tilapia was the most prone to lipid oxidation. Free fatty acid composition decreased as the storage time was extended. The activity of LOX was relatively high during storage, suggesting that it could be an important enzyme to catalyzing lipid oxidation during storage. The majority of volatile compounds changed during ice storage. According to the PCA, 2-butanone and nonanal in muscle, 6-methyl-2-haptanone and 2-nonanal in gill, and 1-haptanol, and 1-nonanol in skin showed potential to be used as freshness indicators. In addition, hexanal was a potential marker for measuring degree of lipid. To preserve the quality of tilapia, gills should be removed prior to ice storage and filleting.

Goatfish, lizardfish, and threadfin bream are major raw materials for tropical surimi production. They high concentration of polyunsaturated fatty acid (PUFA) susceptible to lipid oxidation. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are the two most abundant PUFA in all tropical fish studied, with the highest concentrations observed in gill. Goatfish gill showed the highest PUFA. Lizardfish gill exhibited the highest LOX activity. LOX activity of lizardfish surimi processing decreased from 157.19 U/g in raw material to 57.85 U/g in surimi. Lizardfish surimi contained a high amount of DHA and EPA, indicating that LOX could partly contribute to lipid oxidation during surimi production and frozen storage. Optimal activity of lizardfish gill LOX was at 25 ° C and pH 7. 5. The enzyme was stable at temperatures below 50 ° C. Lizardfish gill LOX exhibited substrate specificity toward EPA. The enzyme was completely inhibited by 1 mM ethylenediaminetetraacetic acid (EDTA) but activated by 1 mM Fe^{2+}, Na^{2+}, and Ca^{2+}.

BIOGRAPHY

Wilaiwan Karbsri was born in April 22, 1990, at Chiang Mai, Thailand. She studied for her high school diploma at TheeraKarnBanhong School, Lamphun, Thailand (2003-2008). In 2012, she received Bachelor's degree of Science (Food Technology) from Suranaree University of Technology. In 2013, she received SUT-PhD scholarship program (SUT-PhD/03/2556), Suranaree University of Technology, Nakhon Ratchasima, Thailand to study her Ph.D. program in Food Technology.

She presented poster presentation including: 1) The 4th SUT International agricultural colloquium (Suranaree University of Technology, Thailand, 28-29 June, 2016) with the poster presentation in title of "The role of lipoxygenase in lipid oxidation of Nile tilapia (*Oreochromis niloticus*) during ice storage", 2) The 19th Food Innovation Asia Conference (Bangkok, Thailand, 15-17 June 2017) with poster presentation in title of "Lipoxygenase-catalyzed lipid oxidation of Nile tilapia (*Oreochromis niloticus*) and volatile compounds formation during ice storage", 3) The 5th SUT International agricultural colloquium (Suranaree University of Technology, Thailand, 15-16 August, 2017) with the poster presentation in title of "Purification and characterization of lipoxygenase from lizardfish (*Saurida tumbil*)".

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