Doctoral Dissertation

STUDY OF THE UNPLEASANT SMELL IN RABBIT FISH *Siganus fuscescens*: THE GENERATION MECHANISM AND METHOD FOR REMOVAL

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Contents

Abstract

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Abbreviations

ARA	Arachidonic acid
CAR	Carboxen
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DMOX	4, 4-Dimethyloxazoline derivatives
DVB	Divinylbenzene
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectroscopy
LA	Linoleic acid
LOX	Lipoxygenase
LRI	Linear retention index
MUFA	Monounsaturated fatty acid(s) (monounsaturates)
OAV	Odor activities value
PDMS	Polydimethylsiloxane
PUFA	Polyunsaturated fatty acid
SFA	Saturated fatty acid(s) (saturates)
SPME	Solid-phase microextraction
VLOP	Volatile lipid oxidation product

Chapter 1 : Introduction

Rabbit fish (*Siganus fuscescens*), a herbivorous marine fish widely distributed in Japanese waters, is an unpopular fish with a very low commercial value due to the unpleasant smell in its meat. The custom of eating rabbit fish is also limited to only some local area in Japan. The limited fishing of this species consequently has the negative impacts on the productivity of the Japanese shoreline because of the imbalance in fish stock. Moreover, the excessive consumption of macroalgae by its overpopulation has been considered as one of the significant causes involved in the reduction of seaweed beds, which seriously occurred not only in coastal areas of Japan, but in warm temperate waters around the world as well. Although, many studies revealed the potential use of this fish as a raw material in the food industry, the utilization of this fish is still limited. Understanding its smell generation may provide a useful information for smell quality improvement, which probably lead to its wider utilization.

It has been generally accepted that smell is one of the most important factors, which significantly impact the consumer acceptance of fish. The volatile compounds contributing to the smell of fish could be classified into three groups base on their origin, including fresh fish odor, oxidized odor and microbial spoilage odor. The smell of fresh fish may vary among species, but newly caught fish usually contains low level of volatile compounds, which is nearly odorless or has a mild and delicate smell. The major odor-impact compounds in fresh fish commonly are 6-, 8- and 9-carbon carbonyls and alcohols, which usually represent a green, plant-like, and melon-like flavor notes. After harvest, the development of fish smell is dominated by many factors, such as autolytic activity, endogenous enzyme activity and lipid oxidation. Among these factors, lipid oxidation is appeared to play an important role on fish smell generation due to the fact that fish is rich in PUFA, so it is very susceptible to lipid oxidation, which lead to the formation of volatile lipid oxidation compounds, such as hexanal,

1-octen-3-ol, 2,4-heptadinal with an oxidized, fishy, stale, and rancid smell. Furthermore, microbial metabolism by specific spoilage organism also contributes to the change of fresh fish smell. The mainly compounds that represent a sour, putrid ammonia-like of spoilage smell are trimethylamine, dimethyl sulfide and 3-methyl-1-butanol.

Gas chromatography – mass spectrometer (GC-MS) is generally used for analysis of volatile compounds due to its high separation powers and sensitivity. Several headspace sampling techniques have been developed to extract the volatile compounds from sample before gas chromatography analysis, such as simultaneous steam distillation with solvent extraction, static headspace and dynamic headspace techniques. Solid-phase microextraction or SPME is an alternative extraction technique developed in early 90s by Pawaliszyn and co-workers, which combines the sample preparation and sampling into one step. SPME is an economical and solventless methods. Because of its high sensitivity and selectivity, SPME provides many advantages over other techniques, so it has been widely used for analysis of volatile compounds in several food, including seafood. In this study, to clarify the key volatile compounds that contribute to the unpleasant smell of rabbit fish tissues, SPME-GC-MS technique was proposed.

Lipoxygenase (LOX, EC 1.13.11.120) has been well established as enzyme that plays important role on the smell development in plants and animals. This enzyme is capable to catalyze the oxidation of PUFA containing Z,Z-1,4-pentadiene units to produce conjugated unsaturated fatty acid hydroperoxides, which then breakdown to form lower-molecular weight secondary products that are responsible for the smell generation process. In 1984, LOX was firstly reported to be responsible for the generation of fresh fish flavor and aroma in emerald shiners due to their ability to produce the specific carbonyls and alcohols without the corresponding production of random oxidation volatile products. Since then, LOX has been

reported in tissues of several fish species, such as ayu and silver carp, with the involvement in the formation of both desirable and undesirable smell. Because the types and concentration of LOX vary depending on fish species, the volatile compounds that enzymatically generated via LOX might be different as well. Therefore, to examine the genesis of the unpleasant smell in rabbit fish, the volatile compounds formed via lipid oxidation model of crude LOX were demonstrated in our study.

In the production of fish protein gel, such as surimi products, the essential step used for the gel quality improvement is washing. This step could remove not only the impurities in fish meat, but also the water-soluble protein, mainly sarcoplasmic protein including heme protein and various metabolic enzymes that impede the gel-forming ability of surimi during storage. Recently, washing has been reported to affect the overall smell of fish mince by washing away the off-flavor or facilitating the release of other volatile compounds. Furthermore, adding antioxidants either in grinding step or washing solution has successfully prevented the lipid oxidation in fish. Therefore, in the last chapter, the effect of washing with antioxidants solution on the quality of rabbit fish meat during washing and storage was investigated with special focus on the removal of volatile compounds. This method is expected to be an effective method for preventing or decreasing the unpleasant smell in rabbit fish meat, which lead to the increase of their potential use for manufacturing, and be a successful method for managing and restoring seaweed beds.

1.1 Objectives

This research aims to develop the method for preventing or decreasing the unfavorable smell in rabbit fish meat to its wider utilization for food manufacturing, which may be a potential method for managing and restoring seaweed beds. The objectives of this study were as follow.

(1) To characterize the key volatile compounds contributing to the unpleasant smell in rabbit fish tissues using SPME-GC-MS technique

(2) To determine the lipid content and fatty acid compositions in the rabbit fish tissues

(3) To examine the enzymatic generation mechanism of the volatile compounds via lipid oxidation model of crude enzyme and PUFA

(4) To develop the washing method for removing the volatile compounds contributing to the unpleasant smell in rabbit fish minced meat

Chapter 2 : Literature review

2.1 Rabbit fish

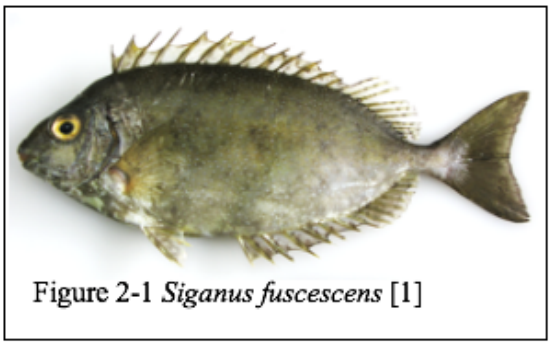

Figure 2-1 *Siganus fuscescens* [1]

Rabbit fish *Siganus fuscescens* (Houttuyn, 1782), a herbivorous marine species, is a tropical Perciform fish in the family Siganidae, which inhabit the shallow waters in the Indo-Pacific. This Siganidae family have a single genera *Siganus* that characterized by having pelvic fin with 3 soft rays between an inner and outer spine with well-developed poison glands. The maximum length of rabbit fish is about 40 cm, while the color of its body is olive green or brown with small spots [1]. Rabbit fish is a herbivorous species having brown algae such as *Sargassum* as its main food sources [2–5], but some small amount of sessile animals and zooplankton also could be found in the stomach and intestine contents of adult rabbit fish as its important nutritional source [3, 4]. This evidence also has been found in several juvenile and adult herbivorous fish [6], suggesting the difference in the feeding behavior among herbivorous species.

In Japan, rabbit fish is one of common species, which can be caught off shore years round. However, this fish is unpopular, having a very low commercial value due to its poisonous spine and the unpleasant smell [7]. Therefore, the custom of eating rabbit fish in Japan is limited to only some local area, such as in Awaji island and Wakayama prefecture [8, 9]. On the other hand, rabbit fish has a high market value, being a preferred local and natural food in some Southeast Asia countries [9, 10]. For instance, in Philippine, both fresh and dried rabbit fish are one of the most important fish foods, while salt-fermented rabbit fish paste is also popular and could be used as the food condiment [10].

2.1.1Rabbit fish and seaweed reduction phenomenon (Isoyake) in Japan

Seaweed beds are the communities of large benthic plants forming rich marine forest. They have an significance part in the marine food webs and the ecosystem of coastal areas by providing the food sources and habitat to marine life, and serving as the nursery grounds for marine species, such as egg-laying sites and feeding grounds of fish and shellfish [11]. They are also known to have a positive impact on the eutrophic water by absorbing the nutrients and organic materials. Moreover, seaweed beds have been reported that they may be an important source of carbon fixation by absorbing carbon dioxide and discharging oxygen via their photosynthesis activities [12].

Although seaweed beds have significance to global ecosystem, and also important communities to maintain fishery resource. The diminishing of seaweed beds (Isoyake in Japanese) has become a serious problem occurred in most coastal area of Japan [8, 13] and in warm temperate waters around the world as well [14]. About 6400 ha of seaweed beds has disappeared from Japanese coastal area since the period between 1978-1992 [15]. Moreover, it is estimated that more than 40% of the kelp (*Ecklonia* spp.) and fucoid (*Sargassum* spp.) beds in Southern Japan has lost since 1990s [16] (Fig 1-2).

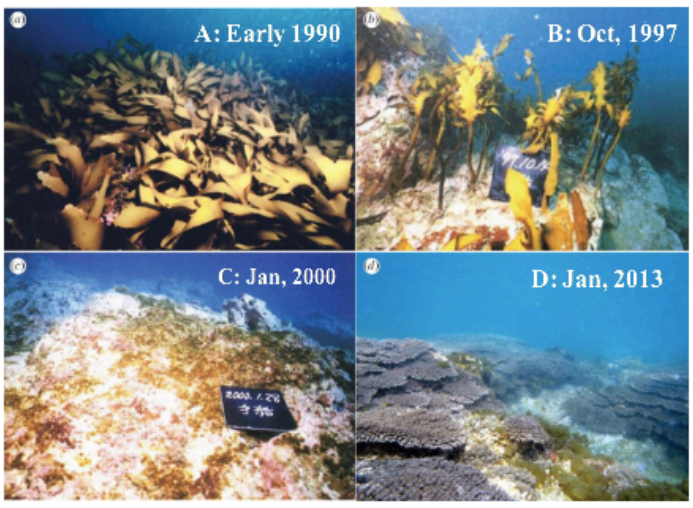

Figure 2-2 Seabed photographs of Tosa Bay (Southern Japan) [14]

Many incidences have been reported to affect the seaweed beds reduction, such as changes in oceanographic and climatic conditions, deficiency of nutrients, grazing or browsing by herbivorous species and outflow of freshwater [13]. However, the survey by Kuwahara *et al*. [17] in 2005 to local governments in coastal prefecture in Japan revealed that overgrazing by herbivores, such as sea urchins and herbivorous fishes, is the most common cause involved in the reduction of seaweed beds.

Several tropical/subtropical herbivorous fishes commonly found in Japanese water, such as rabbit fish, sea chubs (*Kyphosus* spp.) and Japanese parrotfish (*Calotomus japonicus*) (Fig 1-3), have been identified as the significance herbivorous species that responsible for the overgrazing of seaweed beds [18–20]. Among these species, only rabbit fish is recorded to be found from all over the warm temperature, damaging the seaweed in southern to central coasts of Japan [13]. Moreover, because rabbit fish has a very low commercial value in Japan [7], the limited fishing of this species also consequently has the negative impacts on the productivity of the Japanese shoreline because of the imbalance in fish stock [18, 19, 21].

Figure 2-3 Herbivorous fishes mainly involved in the seaweed beds reduction in Japan [22]

2.1.2 The utilization of rabbit fish and the seaweed beds management

The effective use of rabbit fish meat probably leads to its wider utilization, which may be a potential method to manage and restore seaweed beds [8]. Osako *et al*. [23] revealed the high nutrition in rabbit fish meat, especially n-3 and n-6 PUFA, and suggested the potential utilization of this fish as a raw material in fish sauce [24] and fish gel product industry [25]. However, the information about the unpleasant smell of rabbit fish is relatively scarce. Understanding its smell generation may provide a useful information for smell quality improvement and probably lead to its wider utilization.

2.2 Fish lipids

Marine fish are generally known as a major source of PUFA. Their lipids characteristically contain high levels of n-3 PUFA, especially DHA and EPA, which originate from phytoplankton through the marine food chain [26]. High levels of these DHA and EPA were reported in various marine fish species, such as mackerel scad (*Decapterus macarellus*) [27], sardine (*Clupea fimbriata*) [28], tuna (*Scomber australasicus*) [29], Caesioninae species (*Caesio diagramma*, C. tile), Siganidae species (*Siganus canaliculatus*) [30], and bonito (*Euthynnus pelamis*) [31]. On the other hand, only trace amounts of n-6 PUFA, such as AA, are detected in marine fish [26]. However, there are several other marine species that contain comparatively high levels of n-6 PUFA in their lipids, including some kinds of algae [32, 33] and herbivorous species such as abalone [34] and oyster [35].

2.2.1High levels of n-6 PUFA in herbivorous fish

Recent studies have found that herbivorous fish also contain comparatively high levels of ARA [30, 36–38], and carnivorous fish that feed on sea urchins and sea snail, which consume microalgae, contain high levels of ARA as well as DHA [39]. Moreover, besides the high levels of DHA, surprisingly high levels of ARA have also been observed in top carnivorous predatory fish coral reefs, eating mainly small young and juvenile herbivorous fish such as Siganidae species [40]. This suggests that the high ARA levels might be the characteristic of herbivorous species, which may originate from ARA-rich macro-algae.

2.2.2Importance of lipid in fish flavor

Because fish lipid generally contains a significantly high level of polyunsaturated fatty acid, such as n-3 PUFA, which is very susceptible to the oxidation, the degradation of these lipid both from autoxidation and enzymatic reaction generates the volatile compounds that contributed to the smell of fish and seafood products. Complex mixture of these volatile lipid derived products, such as some alcohols, aldehydes and ketones, add the characteristic flavor notes to the fish and seafood products. Fish lipids are responsible for both desirable and undesirable flavor in fish and fish products. It might affect the desirable smell of freshly harvested fish or might results in the deterioration of flavor and the development of the off-flavor [41]. Further information about fish smell and its generation are discussed in chapter 1.4 and 1.5.

2.3 Smell

Smell is very complex sensation. More than 7,000 of volatile compounds have been identified in food [42]. Each volatile compound represents the different smell. For example, isoamyl acetate has a strong odor that could be described as banana-like smell, while 1-octen-3-ol are characterized by mushroom-like smell. Moreover, some food such as coffee, has very complex aromas system, which contains more than 800 volatile compounds. However, the contribution of each compound to aroma perception depends on its concentration and sensory threshold. The odor detection threshold could be defined as the lowest concentration of certain compound that is perceivable by the sense of smell of human [43]. The lower level of threshold indicates the more sensitive of the human olfactometry to those volatiles. For instance, an odor detection threshold of 2-isobutyl-3-methoxypyrazine in water is 0.002 ppb, while those of ethanol is 100,000 ppb in water [44]. The ability of each person to detect odors also affects by many factors, such as experience, genetic variability, olfactory adaptation, and also unpredictable factors such as temperature and humidity [45].

Food generally contains very low concentrations of odor compounds (ppm or even ppb). The difference in the physicochemical properties of these odor compounds, such as volatilities, solubilities, polarities, thermal and pH stabilities, as well as the complexity of food matrices cause the interference with the isolation techniques [46]. Therefore, there is no single and simple method for the identification of smell. Moreover, in case that the sample is concentrated enough, it can be directly injected into the column of gas chromatography (GC), such as essential oil or plants extraction, but if the sample is too dilute or contained within food matric, the isolation technique before GC analysis is needed.

2.3.1 Isolation of volatile compounds

Many techniques have been developed for isolation of aroma chemicals. Each method has a difference in efficiency, reproducibility and range of sample that it can be used. Depending on the principle of aroma compounds isolation, sample preparation methods before GC analysis can be separated into 4 groups, including solvent extraction methods, steam distillation methods, headspace techniques and sorptive techniques.

1.2.1.1 Solvent extraction methods

Solvent extraction methods are the most widely used, which originate from organic chemistry extraction. The concept of these methods is to transfer the volatile chemicals from the sample to organic solvent, by mixing the sample with organic solvent, allowing separation, collecting the solvent phase, and then removing solvent by evaporation. This method shows the most accuracy quantitative of volatile compounds among all isolation methods and also relatively easy to perform. However, these methods need large amounts of sample, and solvent removal step may cause the loss of some volatile compounds. Moreover, solvent peak may cover the early eluted compounds in chromatogram. Many solvent extraction techniques have been developed and used for analysis of volatile compounds in both plant and animal origin food, including seafoods, such as accelerated solvent extraction [47, 48], supercritical fluid extraction [49, 50], and solvent assisted flavor evaporation (SAFE) [51, 52].

1.2.1.2 Steam distillation methods

The concept of the simple steam distillation is to heat the sample which dispersed in water either directly or indirectly by steam. After collecting the condensed vapors, analyst is then extracted with organic solvent. The main advantage of this technique is that the resulted extractions do not contain any contaminant of non-volatile compounds. However, this method

is not suitable for fresh sample because the thermal process is affected the smell of sample by changing the smell to cooked aroma rather than fresh aroma.

1.2.1.3 Headspace-sampling techniques

Headspace collection is probably the easiest technique to extract the volatile compounds among all techniques described above. It is solventless technique, with a convenient, simple, and require only small amounts of sample. Moreover, it is able to be used for any types of sample. However, the relative concentration of resulted compounds extracted from these techniques may not reflect the real concentration in sample because of the differences in the volatility of aroma compounds. Headspace techniques can be divided to static headspace and dynamic headspace (purge and trap). These two techniques are one of the most common techniques used for analysis of volatile compounds in many foods, including fresh fish and fish products [53–55].

1.2.1.4 Sorptive techniques

Sorptive techniques are also rapid and solventless techniques, which combine both sample preparation and extraction of volatile compounds. Solid-phase microextraction or SPME is one of the sorptive techniques, which developed in early 90s by Pawaliszyn and co-workers [56]. Due to its high sensitivity and selectivity, SPME provides many advantages over other headspace sampling techniques, and has been widely used for analysis of volatile compounds in several food [57, 58]. However, SPME technique require a careful selection of several parameters, such as type of fiber, the extraction time and temperature. A fused silica fiber coated with polymeric film are used for absorbing the volatile compounds from the headspace of sample, which is placed in headspace vial and sealed with a septum cap (Fig 1-4a). After fixing sampling time, SPME fiber is directly desorbed into GC or GC-MS injector port (Fig 1-4b).

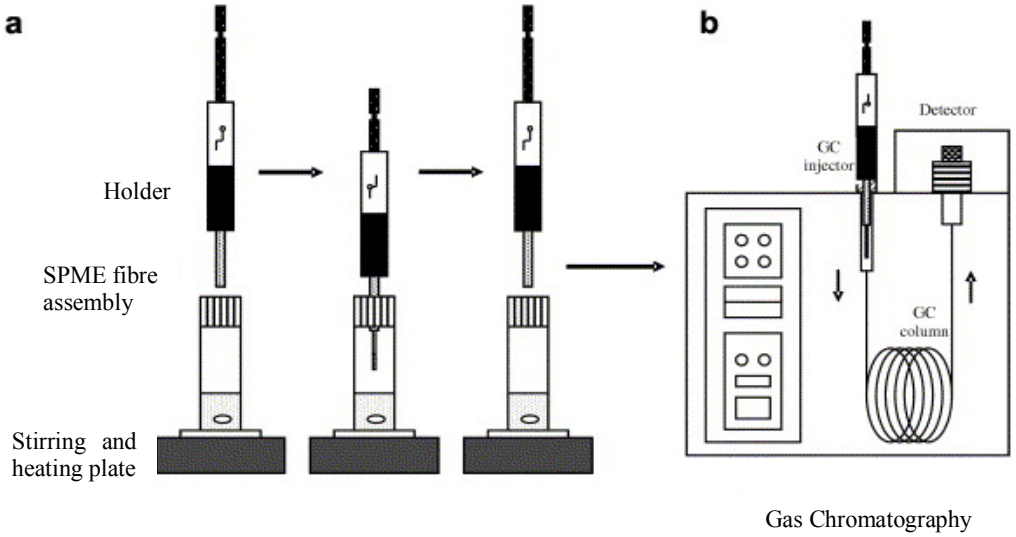

Figure 2-4 Solid-phase microextraction (SPME) technique (Reprinted with permission from [59])

2.3.2 Analysis of volatile compounds

Gas chromatography (GC) is used for the separation of complex aroma mixtures into individual chemical components based on the differential migration of each compounds through a stationary phase in a mobile phase at high temperature. The stationary phase in gas chromatography is a piece of glass or metal tube called column, which consists of a microscopic layer of liquid or polymer on an inert solid support inside, while the mobile phase is a carrier gas, usually an inert gas such as helium. Many types of detectors have been used with GC, such as flame ionization detector (FID), flame photometric detector (FPD), and thermal conductivity detector (TCD). Among these, mass spectrometer (MS) is the most widely detector for analysis and identification of flavor compounds [60] (Fig 1-5).

Figure 2-5 Gas chromatography – mass spectrometer (GC-MS) [61]

Moreover, to confirm the identify of volatile compounds, linear retention times or retention indices (LRI), which based on calculating the retention time of compounds with those of n-alkane series eluting before and after compounds as shown in equation 2-1, could be used.

$$LRI = \left(\frac{Rt_{(x)} - Rt_{(n)}}{Rt_{(n+1)} - Rt_{(n)}} + n\right) \times 100 \qquad \text{(equation 2-1)}$$

$Rt_{(x)}$ is the retention time of each volatile compound (x), while $Rt_{(n)}$ and $Rt_{(n+1)}$ are retention times of n-alkanes eluting directly before and after the compound (x).

2.3.3 Volatile aroma compounds

Large group of compounds have been reported in food. These compounds can be classified into classes based on their chemical structure and general properties [62], including alcohols, aldehydes, ketones, esters, acids, hydrocarbons, cyclic compounds, phenol, sulfur-containing and nitrogen-containing compounds.

2.3.4 Key odor compounds in food

Although, foods contain a very large number of volatile compounds, not all of those are contributed to the overall smell of foods. The odor activity value (OAV) were proposed by Patton and Josephson [63]. They suggested the definition of this value by calculating the ratio of the concentration of compounds in food to its sensory threshold in that food. The compounds that occur above their sensory threshold are likely to be significant contributors to the overall aroma, while those presenting below threshold are not.

2.4 Smell of fish

It has been generally accepted that smell is one of the most important factors, which significantly impact the consumer acceptance of fish. The volatile compounds contributing to the smell of fish could be classified into several groups based on their origin, including fresh fish odors, species-specific odors, deteriorated odors, process-induced odors and environmental derived odors.

2.4.1 Fresh fish odors

The smell of fresh fish may vary among species, but newly caught fish usually contains low level of volatile compounds, which is nearly odorless or has a mild and delicate smell [64]. The major odor-impact compounds in fresh fish commonly are 6-, 8- and 9-carbon carbonyls and alcohols, which usually represent a green, plant-like, and melon-like flavor notes [65]. Six-carbon volatile compounds, such as hexanal, represent the distinctly green plant-like odor, are mainly observed in freshly harvested fish. Eight-carbon volatile alcohols and ketones, including 1-octen-3-ol, 1-octen-3-one, 1,5-octadien-3-ol, 1,5-octadien-3-one, have been identified in most fish, shellfish and crustacean species. Although these compounds are

individually characterized by a mushroom-like smell, they appear to represent the distinct heavy, fishy and grassy smell when it contributes to the fresh fish [64]. Nine-carbon compounds contribute to a fresh, green cucumber-like, which provide the specific odor to certain fish species, such as Great Lakes whitefish (*Coregonus clupeaformis*) [66] and ayu (*Plecoglossus altivelis*) [67]. The five-carbon compound, such as 1-penten-3-ol, is also found in fresh fish. However, considering their comparatively high threshold level, it may not likely to be a significant contributor to the characteristic aroma of fresh fish [65].

In general, volatile carbonyls represent heavy and coarse aromas, while the volatile alcohols contribute lighter qualities. Moreover, due to the lower threshold levels of volatile carbonyls, they appear to be more contribute to the overall fresh fish smell than their corresponding alcohols do [65]. The further information of the formation of these volatile compounds associated with fresh fish smells are described in section 1.5.

2.4.2 Species-specific odors of fish

The smell of some fish is distinct and specific to their species. For example, salmon have a rich flavor that can describe as salmon-like, which is considered to be associated with carotenoid pigments and others lipid substance. The carotenoids might relate to the chemical reaction that convert fatty acids to salmon-like flavor. The compound that contribute to the salmon-like aroma is considered to be one of the alkyl furanoid-type [68]. Moreover, some other fish such as ayu also have a characteristic sweet smell of watermelon- or cucumber- like, which are represented by (E,Z)-2,6-nonadienal and (Z)-3-hexenol [67].

2.4.3 Deteriorated fish odors

1.3.4.1 Trimethylamine

Trimethylamine and dimethylamine have been known as compounds contribute to the odor of deteriorating fish. Trimethylamine, which represent an old-fishy-like smell with a very low threshold (300-600 ppb) [69], is a compound generated from microbial reduction of trimethylamine oxide, while dimethylamine and formaldehyde are the breakdown product of trimethylamine oxide by enzyme in the muscle of fish. Therefore, they have been suggested as indicators of spoilage, which could be used for the evaluation of fish freshness [70].

1.3.4.2 Autoxidation related odor

Autoxidizing fish lipids has been related with the presence of fishy smell in stored fish. The oxidation develop the oxidized odor, which contribute to a fishy, cod liver oil-like, which changed from the initially smell described as green or cucumber-like [71]. The most significant compounds contributed to these oxidized smell are (E,Z,Z)-2,4,7-decatrienal and (E,E,Z)-2,4,7-decatrienal as well as hexanal and (E)-2-hexenal, which were reported in various autoxidized fish oil [71, 72]. A 2,4,7-decatrienals are suggested to be derived from autoxidation of long-chain n-3 PUFA, which are the dominant PUFA in fish lipids.

2.4.4 Process-induced odors

Thermal processing also affects the development of flavor in food, including fish. Canned tuna possesses a characteristic meaty smell from the producing of 2-methyl-3-furanthiol by reaction between ribose and cysteine [64, 73]. Moreover, dried, smoked, pickled and fermented fish also have their own characteristic smell.

2.4.5 Environmentally derived odors

Environment is also the significance factor affects the flavor of fish. The two major compounds responsible for off-flavor from environment are geosmin and 2-methylisoborneol, which represent a musty muddy, earthy and mouldy odor in various wild and farmed fish [74–76]. These 2 compounds are secondary metabolites produced by various cyanobacteria and actinomycetes in the water [77–79], which can be absorbed to fish through the gills, skin, small intestine and stomach of fish [80].

2.5 Lipoxygenases and smell formation in fresh fish

2.5.1 Lipoxygenase

Lipoxygenase (LOX, EC 1.13.11.120) has been well established as enzyme that plays important role on the smell development in plants [81] and animals [82, 83]. This enzyme is capable to catalyze the oxidation of PUFA containing Z,Z-1,4-pentadiene units to produce conjugated unsaturated fatty acid hydroperoxides [81], which breakdown to form lower-molecular weight secondary products that are responsible for the smell generation process in fresh fish.

2.5.2 Physicological role of lipoxygenase

The information on the role of LOX in fish is limited. Josephson and Lindsay [65] suggested the possible role of LOX that may produce physiologically active, such as slime secretion regulation, or osmoregulatory compounds, such as leukotrienes and hydroxy fatty acids, from PUFA. These compounds could inactivate by hydroperoxide lyase. In addition, it

has been reported that the rate of volatile carbonyls and alcohols formation appears to relate with stress, which is higher in the gills, skin and mucus layer of the fish than in the muscle.

2.5.3 Substrate specificity

LOX has an ability to dioxygenate a specific position on PUFA possessing 1,4-Z,Z-pentadiene moieties. The dominant n-3 PUFA, including eicosapentaenoic acid (EPA) and docosahexanenoic acids (DHA) observing in fish also serve as a common substrate for LOX. In addition, some fish also contain detectable levels of n-6 PUFA, arachidonic acids (ARA), which could be a substrate of LOX as well. The clear difference between LOX-initiated peroxidation and the autoxidation is that the high stereospecificity of the enzyme-mediated oxygen addition.

2.5.4 LOX and the generation of smell in fresh fish

As describe above in section 1.3.1, freshly harvested fish usually possess a green, plant-like, and melon-like flavor [65], which associated with the major volatile compounds, including 6-, 8- and 9-carbon carbonyls and alcohols. Josephson et al. [84] firstly reported the presence of LOX in fish, which is responsible for the generation of these specific volatile compounds associate with the smell in fresh fish. Since these early investigations, LOX activity has been reported in other several species of fish [65, 85–87]. Up to now, it could be suggested that the different type and concentrations of volatile compounds contribute to fresh smell of various fish are caused by the different type and concentration of LOX as well as available substrate (PUFA) in its tissues.

The main LOX involve in the biogeneration of smell in fresh fish are 12- and 15-LOX and hydroperoxide lyase. The proposed mechanism of the generation of some selected volatile compounds in fresh fish initiated by LOX are shown in figure 1-1 and 1-2. The 12-LOX, which act on specific PUFA, produce n-9 hydroperoxides, which the hydrolysis of these 9-hydroperoxide of EPA by hydroperoxide lyase leads to the formation of (Z,Z)-3,6-nonadienal. These compounds may spontaneously or enzyme-catalyzed undergo to their isomeration, (E,Z)-2,6- nonadienal, and change due to the reduction to their corresponding alcohols. The aldehydes conversion to their corresponding alcohols is a significant step due to the fact that alcohols have somewhat higher odor threshold than aldehydes, so the overall smell intensity is decreased. The act of LOX on n-6 PUFA, ARA, involve in the formation of 8-carbon ketones and alcohols, including 1-oncten-3-ol and 1-octen-3-one, which is one of the major compounds contribute to the smell of fresh fish. Alcohols is converted to their corresponding ketones be the action of nonspecific dehydrogenase. This step has a significantly effect on the fish aroma because 1-octen-3-one have much lower odor detection threshold than 1-octen-3-ol.

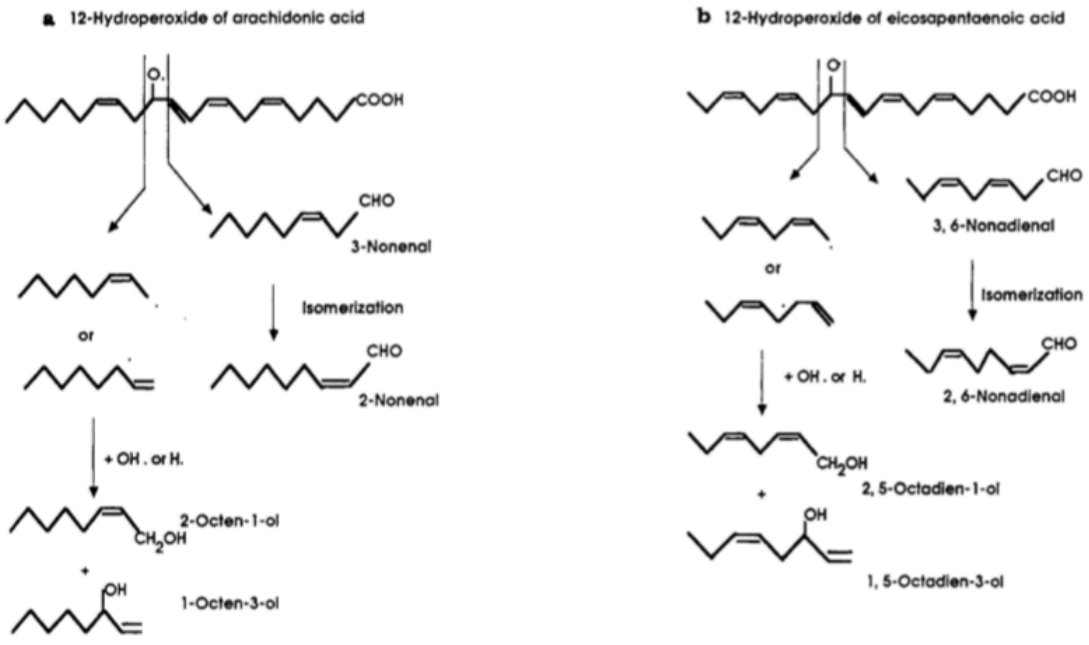

Figure 2-6 The proposed mechanism of the generation of some selected volatile compounds in fresh fish initiated by LOX. (Reprinted with permission from [86])

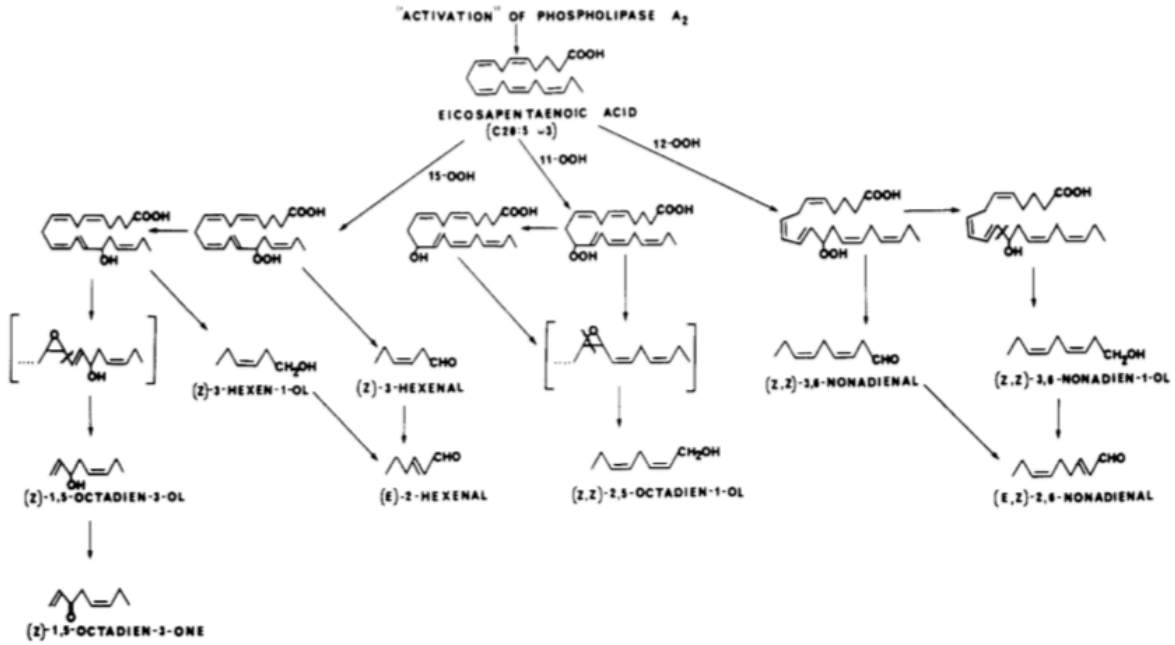

Figure 2-7 The proposed mechanism of the generation of some selected volatile compounds in fresh fish initiated by LOX. (Reprinted with permission from [84])

2.6 Method for preventing or reducing the unpleasant smell in fish and seafood products

The occurrence of the unpleasant smell in fish is affected by many factors. As describe above in section 1.4, the off-flavor might be related to the environmental conditions, composition, physiological and biological state of fish, processing methods and storage conditions, [88]. Several methods have been developed in order to alleviate problem of the off-odor generation in fish and seafood products. Most of them are based upon one or several of these following three approaches [88].

2.6.1 Masking the off-odor by addtion of substance or by processing

The unpleasant smell could be masked by two techniques. First is by adding the ingredients that capable to mask the off-odor, such as variety of herbs and spices [89–91]. Kikuchi *et al.* [89] reported the suppression of trimethylamine odor after mixing with some spices, such as onion, laurel, and sage, which is considered to be due to some chemical reaction between those compounds and the spices. The second techniques that could be used for masking the off-odor is by some processing, such as smoking. The unpleasant smell of sardine could not be detected by the most of sensory panelists in smoked sardine comparing to raw, and dried sardine [92].

2.6.2 Elimination of the off-odor

This method deals with the removal of the cause of the off-odor prior to processing. For instance, in order to prevent the occurrence of compounds originated from environment of fish, including geosmin and 2-MIB, grow-out environment of fish should be concerned. Some techniques, including minimize the feed waste and increase the turbidity of pond water to suppress the growth of phytoplankton, could be used [93].

On the other hand, the method that could be remove the lipid pro-oxidants, oxygen, or other compounds susceptible to oxidation, could be used to reduce the unpleasant smell generated via lipid oxidation [94]. One of a common technique used in the production of fish protein gel, such as surimi products, is washing. Washing step has been reported to affect the overall smell of washed silver carp (*Hypophthalmichthys molitrix*) meat by washing away the off-flavor or facilitating the release of other volatile compounds [95]. Washing is the essential step used for the gel quality improvement because it could remove not only the impurities in fish meat, but also the water-soluble protein, mainly sarcoplasmic protein including heme

protein and various metabolic enzymes that impede the gel-forming ability of surimi [96]. Washing step has been recently confirmed to have an impact on lipid oxidation of washed horse mackerel (*Trachurus trachurus*) meat, resulting in the less formation of VLOP, such as 1-penten-3-ol and 2,4-heptadienal, during cold storage [97].

2.6.3 Retardation of the development of the off-odor

The development of off-odor in fish could be retarded by several techniques, such as freezing [94, 98], irradiation [99, 100], using oxygen barriers [101, 102], or using antioxidants.

Adding antioxidants either in grinding step or washing solution has successfully prevented the oxidation in fatty fish, such as Atlantic mackerel (*Scomber scombrus*) [103, 104] and horse mackerel [105], resulting in the lower level of VLOP, including hexanal and 1-penten-3-ol, in meat washing with antioxidants solution during storage than that in meat washing with water [105].

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Chapter 3 : Determination of the volatile compounds in rabbit fish tissues using SPME-GC-MS technique

3.1 Introduction

Rabbit fish (*Siganus fuscescens*) is a herbivorous marine fish widely distributed in Japanese waters. Excessive consumption of macroalgae by its overpopulation has been considered as one of the significant causes involved in the reduction of seaweed beds, which seriously occurred not only in coastal areas of Japan, but in warm temperate waters around the world as well [1–3]. The effective utilization of this fish probably be one of the sustainable managements for restoring seaweed beds [4]. Many studies reported the high nutrition in rabbit fish meat, especially n-3 and n-6 PUFA [5, 6], and the potential to use as raw material in the fish sauce and textured products industry [7, 8]. However, the major bottleneck for its more extensive utilization as food source, and limiting the custom of eating this fish to only some local areas in Japan, is the presence of the unpleasant smell in its meat [4]. Therefore, understanding its smell profile may provide a useful information for smell quality improvement and probably lead to its wider utilization. To our knowledge, there has been little information about the genesis of unpleasant smell in rabbit fish tissues, except for the study of Miyasaki & Fujioka [9] who investigated the volatile compounds in the meat of several herbivorous species, including rabbit fish.

Solid-phase microextraction or SPME is an alternative extraction technique developed in early 90s by Pawaliszyn and co-workers [10]. Due to its high sensitivity and selectivity, SPME provides many advantages over other headspace sampling techniques, and has been widely used for analysis of volatile compounds in several food, including seafood. However, SPME technique require a careful selection of several parameters, such as type of fiber which determines the specificity of the extraction and the time of extraction. Therefore, in this chapter,

the optimization of these parameters was examined to propose the best condition of SPME in order to extract the volatile compounds from the headspace of rabbit fish tissues. The SPME with the best extraction condition was then used for analysis of volatile compounds in rabbit fish tissues, including muscle, viscera, skin and stomach contents, to clarify the genesis of the unpleasant smell in rabbit fish.

Moreover, volatile compounds in rabbit fish meat during 6 days of cold storage as filleted and as whole fish was also analyzed to compare the difference in the development of volatile compounds contributing to the unpleasant smell of rabbit fish during storage.

3.2 Material and methods

3.2.1 Chemicals

Authentic reagents used for identification of volatile compounds were obtained from Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), Merck (Hohenbrunn, Germany) and Alfa aesar (Heysham, UK).

3.2.2 Fish sampling, preparation and storage

Rabbit fish *Siganus fuscescens* (average fork lengths 33.1 ± 1.5 cm, average body weight 499.7 ± 78.2 g, n=10) were caught off the coast of Nagasaki, Japan (32° 37'N, 128° 45'E) using set nets and transported to our laboratory under ice-storage condition. After arrival, the fish was separated into two groups. The first group were kept as whole fish (W) for storage experiment. The second group was manually dissected into dorsal white muscle, dorsal skin,

and viscera. Stomach contents were separated from viscera and also used as sample. The dorsal meat of this group was used as the fillet (F) for storage experiment.

For storage experiment, whole fish (W) was stored in polyethylene bag at 4 °C for 6 days, and volatile compounds were analyzed every 2 days. Sample was dissected just before volatile analysis, and the white dorsal meat was used as meat sample from whole fish storage sample. Dorsal white meats of the second group fish were stored in polyethylene bag at the same condition as whole fish.

3.2.3 Optimization of the volatile compounds analysis by SPME-GC-MS

A 4 SPME fiber with different types of coated stationary phase, including 50/30 μm DVB/CAR/PDMS, 85 μm CAR/PDMS, 65 μm PDMS/DVB and 85 μm polyacrylate, were used for volatile compound analysis. The effect of extraction time on the SPME performance were also determined by varying the time used for absorbing the volatile compounds in headspace of sample from 15, 30 and 45 minutes. In this experiment, viscera, which has the strongest smell comparing to other tissues, was used as sample. The SPME fiber and the extraction times that provided the highest performance were chosen for analysis of volatile compounds in every experiment in this study.

3.2.4 Analysis of volatile compounds in Rabbit fish tissues

A slightly modified method of Iglesias and Medina, (2008) was used for analysis of volatile compounds in rabbit fish muscle, skin, viscera and stomach contents, by homogenizing 3 g of sample with 8 mL of saturated NaCl solution at 3000 r/min for 2 min (PT 10-35 GT; Kinematica, Lucerne, Switzerland). After centrifuging at 5000 r/min for 10 min, 6 mL of

supernatant was placed in a 20 mL headspace glass vial, and cyclohexanol was added as an internal standard to give a final concentration of 0.1μg/mL. The vial was then flush with nitrogen gas and hermetically sealed with screw cap with silicone septa. A 50/30 μm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) SPME fiber (Supelco, USA) was used for collecting volatile compounds from the headspace above the samples for 30 min at 30°C under magnetic stirring. The fiber was then immediately inserted into gas chromatograph injector for 5 min to desorb the volatile compounds.

The analysis was carried out on a Shimadzu GCMS-QP2010 ultra (Kyoto, Japan) equipped with a SUPELCOWAXTM10 fused silica capillary column (60mm length x 0.25mm i.d. x 0.25 μm thickness; Supelco, USA) using helium with a constant flow at 1.2 ml/min as the carrier gas. The injection port and detector temperature were at 260°C. The oven temperature was programmed as follow: from 40 to 200 °C at 4 °C/min, from 200 to 250 °C at 5°C/min, and kept at 250 °C for 5 min. The mass spectrometer was recorded at 70 eV ionization energy by scanning MS from m/z 40 to 500. Volatile compounds from four replicate samples were analyzed.

3.2.5 Identification and Quantification of volatile compounds

The volatile compounds were identified based on comparing and matching their mass spectra with those reported in the mass spectra libraries (NIST11). The identification was considered when at least 3 from 4 replications of sample containing a peak at same retention time with a higher than 85% similarity to the NIST mass spectrum. When available, some authentic standards were analyzed for MS confirmation.

A series of n-Alkanes (C8-C20, Sigma-Aldrich, Merck KGaA, Germany) was determined under the same condition to obtain linear retention index (LRI) values of the compounds, according to equation 1, and compare with those reported in published literatures or in online standard reference database (https://webbook.nist.gov).

$$LRI = \left(\frac{Rt_{(x)} - Rt_{(n)}}{Rt_{(n+1)} - Rt_{(n)}} + n\right) \times 100 \quad (1)$$

$Rt_{(x)}$ is the retention time of each volatile compound (x), while $Rt_{(n)}$ and $Rt_{(n+1)}$ are retention times of n-alkanes eluting directly before and after the compound (x).

Quantities of each volatile compounds was estimated by internal standardization method [11], by calculating the ratio of its peak area to the cyclohexanol internal standard peak area as presented in equation 2.

$$Relative\ response\ factor\ = \frac{A_x}{A_{I.S}} \times \frac{M_{I.S}}{M_x} \quad (2)$$

A_x and $A_{I.S}$ are the peak area obtained from the total ion chromatograms of each compound and internal standard. $M_{I.S}$ is the amount of internal standard that added into sample before analysis. In this study, cyclohexanol was used as an internal standard without considering the response factors, that is, the response factors were all assumed to be 1. Therefore, the estimated concentration of each of the volatile compounds were express according to equation 3.

$$Estimated\ conc.\ (ng/g_{sample}) = \frac{Peak\ area\ ratio\ (compound/I.S) \times \mu g\ (I.S) \times 1000}{g_{sample}} \quad (3)$$

Odor activity values (OAVs) the of volatile compounds found in rabbit fish tissues were estimated by dividing the estimated concentration of each compound in sample with its odor threshold reported in the literatures. Compounds with OAV equal to or greater than 1 were identified as an odor-active compound that contribute to the overall of smell.

3.2.6 Statistical Analysis

Volatile compounds from four replicate samples were analyzed by one-way ANOVA. The differences between mean values were determined statistically with Duncan's New Multiple Range Test at $p<0.05$. In addition, principle component analysis (PCA) was performed with the software PAST PAleontological STatistics (Version 3.18, Øyvind Hammer, Natural History Museum, University of Oslo)

3.3 Results & dicussion

3.3.1 Optimization of the SPME method

To obtain the best condition of SPME for extracting the volatile compounds from the headspace of rabbit fish tissues, SPME with different types of coated stationary phase, including 50/30 μm DVB/CAR/PDMS, 85 μm CAR/PDMS, 65 μm PDMS/DVB and 85 μm polyacrylate, were used. Chromatograms of volatile compounds observed in rabbit fish viscera with 4 different types of SPME fibers extraction are shown in figure 3-1. The compounds are listed according to table 3-1. The highest number of volatile compounds was found in viscera extracted by 50/30 μm DVB/CAR/PDMS fiber (27 compounds), followed by 65 μm PDMS/DVB (26 compounds) and 85 μm CAR/PDMS (25 compounds), while no volatile compound could be extracted from rabbit fish viscera when 85 μm polyacrylate fiber was used. The 3 SPME fiber, including DVB/CAR/PDMS, PDMS/DVB and CAR/PDMS, showed the ability to extract the similar types of major volatile compounds, thought the intensity of each compound was slightly differed.

In this study, several compounds were considered as the contaminants that was not originated from the fish sample, such as hexane, chloroform and some siloxane derivatives, including hexamethyl cyclotrisiloxane, octamethyl cyclotetrasiloxane, decamethyl cyclopentasiloxane, dodecamethyl cyclohexasiloxane, and hexadecamethyl cyclooctasiloxane (Figure 3-1 and Table 3-1). Several investigators reported that because of the sensitivity of SPME, contaminant compounds are commonly observed in the headspace of samples. Hexane and chloroform, an organic solution commonly used in laboratory, were probably absorbed from the container, while the series of siloxane were suggested to be caused by PDMS in SPME fiber [12, 13]. Thus, all data obtained from volatile analysis in our study were identified regardless of the presence of these contaminant components.

To observe the effect of fiber coating on the extraction of volatile compounds, total peak area, and peak area of some volatile compounds with high intensity, were calculated, and presented in figure 3-2 and figure 3-3, respectively. The highest levels of total peak area ($p<0.05$) was observed in CAR/PDMS fiber, while there was no significantly difference between the total peak area of DVB/CAR/PDMS and PDMS/DVB fiber. CAR/PDMS coated fiber also presented the best result for extracting volatile compounds at low retention time, such as 1-penten-3-ol and 1-penten-3-one. However, this fiber was inability to extract 2 carbonyl compounds with higher retention time, including nonanal and 2-octen-1-ol. In the other hand, DVB/CAR/PDMS fiber showed the good results in a wider range of volatile compounds. Souza and Bragagnolo [14] and Garcia-Esteban et al. [15] were reported the similar results in their study. Therefore, in this study, DVB/CAR/PDMS fiber was chosen for extracting the volatile compounds in rabbit fish.

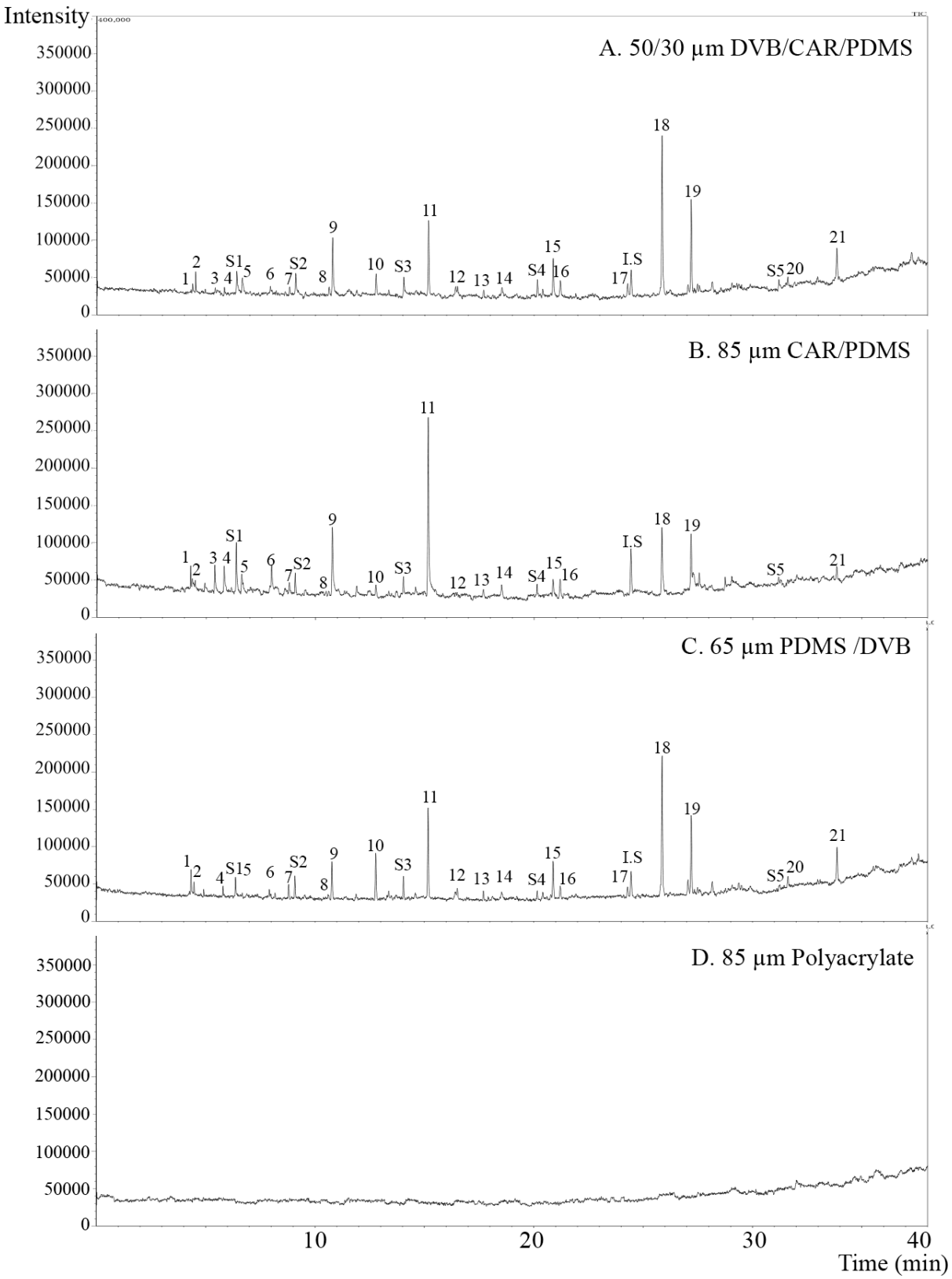

Figure 3-1 Chromatograms of volatile compounds obtained from rabbit fish viscera according to the type of fiber used. Peak number are identified as in Table 3-1.

Table 3-1 Volatile compounds identified in headspace of rabbit fish viscera

Peak number	***Retention time***	***Compounds***
1	4.385	Trimethylamine
2	4.520	Hexane
3	5.270	Carbon disulfide
4	5.430	Dimethyl sulfide
5	6.740	2-Octene
6	7.975	(Z,Z)-3,5-Octadiene
7	8.865	2-Ethyl furan
8	10.630	Chloroform
9	10.845	1-Penten-3-one
10	12.845	Hexanal
11	15.245	1-Penten-3-ol
12	16.625	Heptenal
13	17.790	Unknown
14	18.573	1-Pentanol
15	20.970	1-Octen-3-one
16	21.295	(Z)-2-Penten-1-ol
18	25.955	1-Octen-3-ol
19	27.290	3,5,5-trimethyl-2-Hexene
20	31.715	(E)-2-Octen-1-ol
21	33.925	1-Dodecen-3-yne
Internal standard (I.S)	24.535	Cyclohexanol
S1	6.400	Hexamethyl cyclotrisiloxane
S2	9.100	Octamethyl cyclotetrasiloxane
S3	14.050	Decamethyl cyclopentasiloxane
S4	20.160	Dodecamethyl cyclohexasiloxane
S5	31.205	Hexadecamethyl cyclooctasiloxane

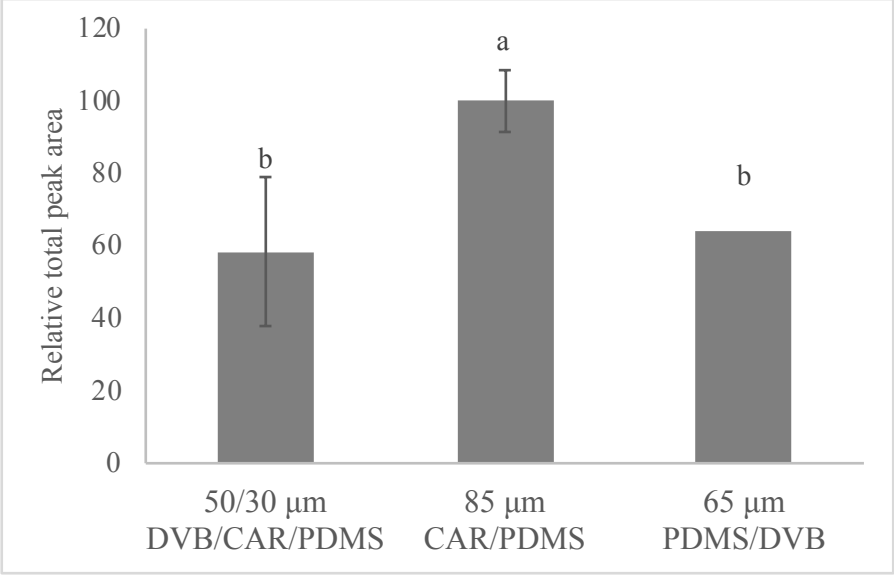

Figure 3-2 Relative total peak area of volatile compounds obtained from rabbit fish viscera by SPME-GC-MS according to the type of fiber used. Different letters indicate statistically significant differences ($p<0.05$).

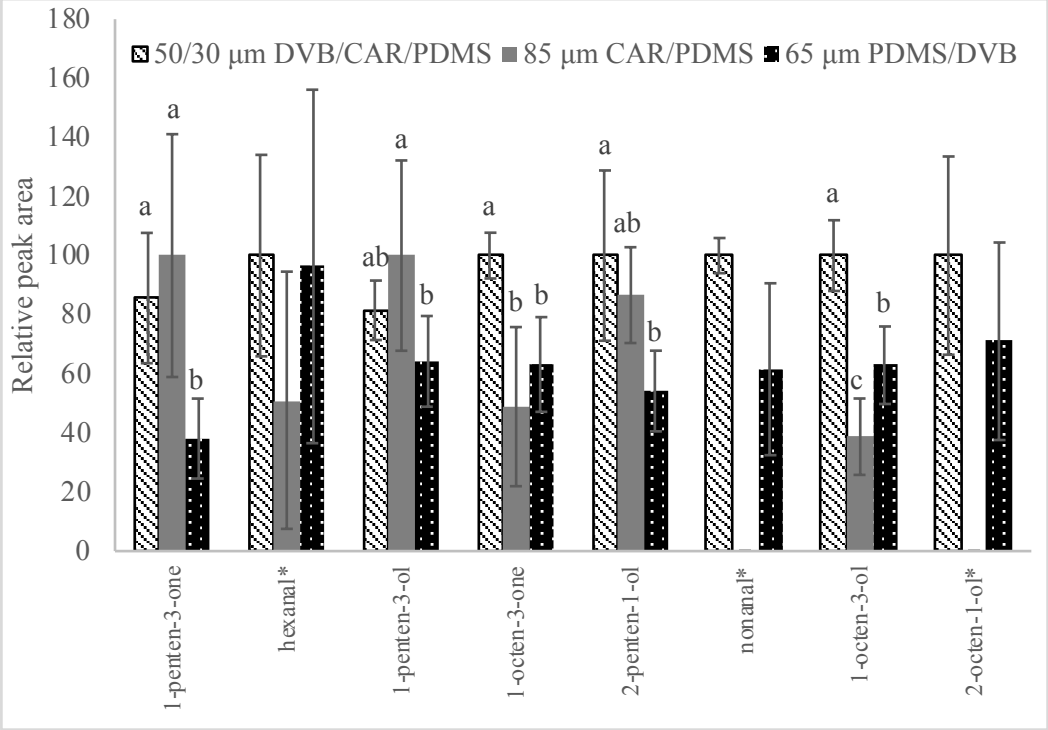

Figure 3-3 Effect of fiber coating on the extraction of selected volatile compounds from rabbit fish viscera. Different letters indicate statistically significant differences ($p<0.05$). * indicates no statistically significant differences.

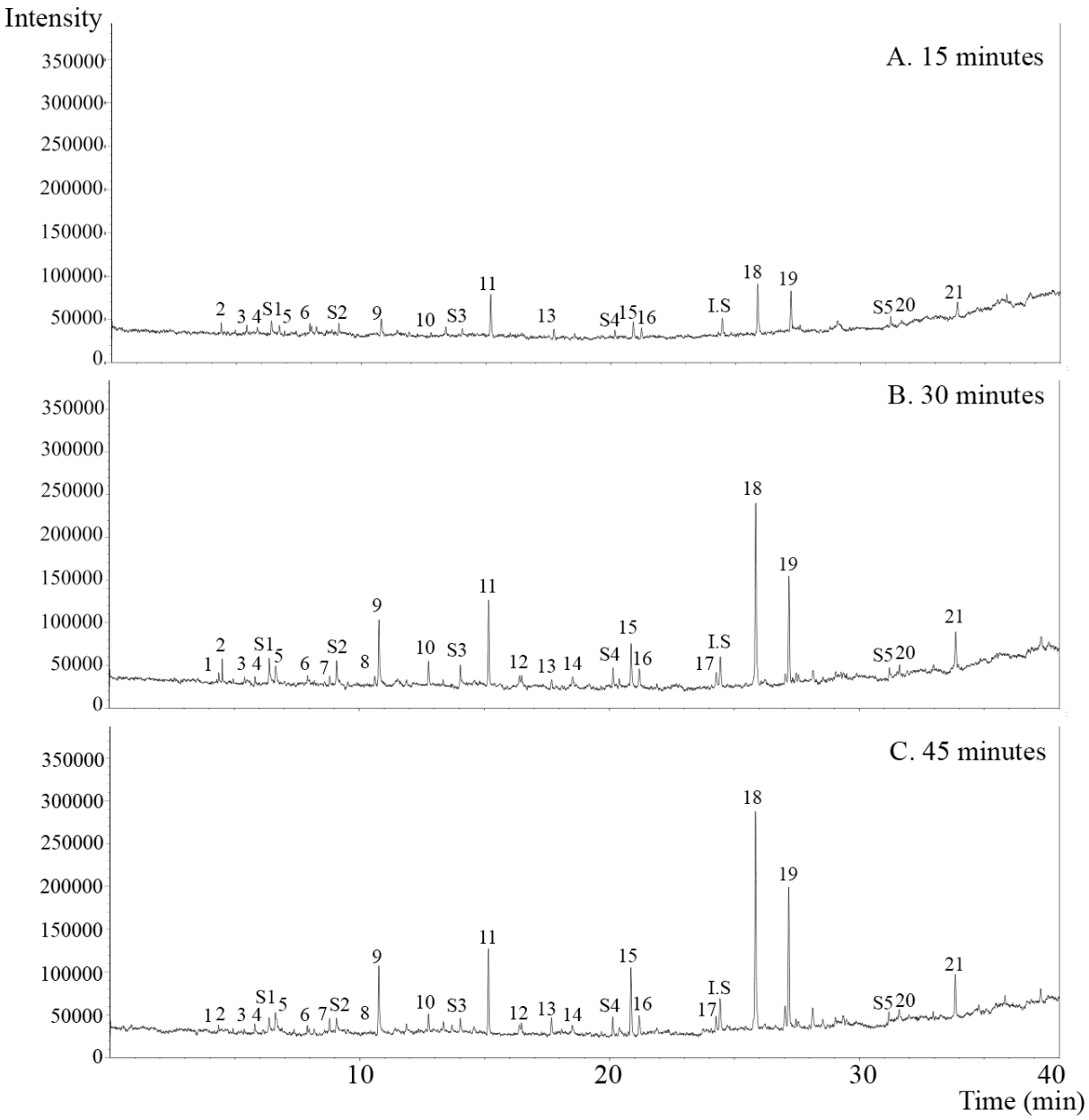

Figure 3-4 Chromatograms of volatile compounds obtained from rabbit fish viscera by SPME-GC-MS according to the extraction times. Peak number are identified as in Table 3-1.

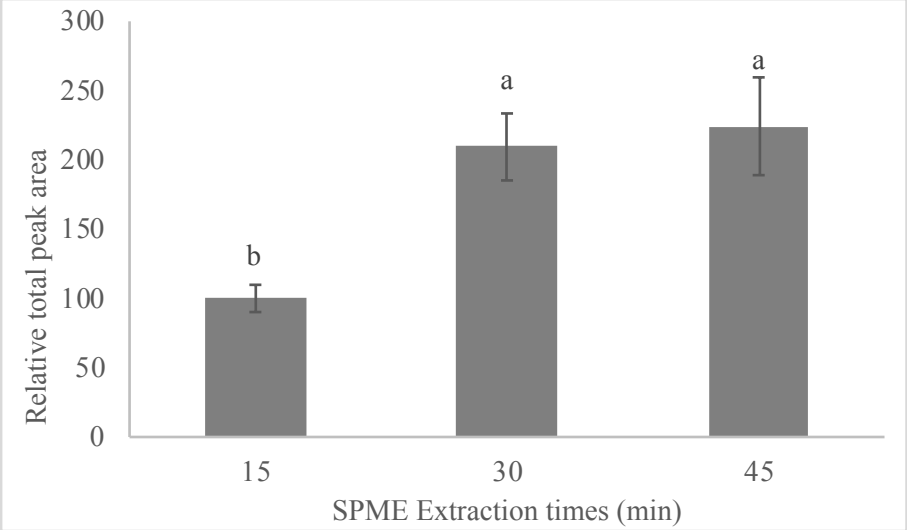

Figure 3-5 Relative total peak area of volatile compounds obtained from rabbit fish viscera by SPME-GC-MS according to the extraction time. Different letters indicate statistically significant differences (p<0.05).

The effect of extraction time was then observed in this experiment. Figure 3-4 shows the chromatograms of volatile compounds obtained from rabbit fish viscera by SPME-GC-MS according to the extraction times. Total peak areas of volatile compounds were calculated, and presented in figure 3-5. Total peak area of compounds tended to increase with the extraction times. However, there is no significantly difference observed between using SPME to extract volatile compounds for 30 and 45 minutes. Thus, an extraction time of 30 minutes were chosen for volatile analysis in this study.

3.3.2 Analysis of volatile compounds in rabbit fish tissues

Figure 3-6 shows a total of 26 volatile compounds detected in rabbit fish muscle, viscera, skin and stomach contents using SPME-GC-MS technique. Peak number are identified as in Table 3-2. The identification was considered when at least 3 from 4 replications of sample containing a peak at same retention time with a higher than 85% similarity to the NIST mass spectrum. Available authentic standards, including trimethylamine, dimethyl sulfide, 3-methyl-butanal, 1-penten-3-one, hexanal, 1-penten-3-ol, heptanal, 1-octen-3-one, (Z)-2-penten-1-ol, nonanal, (E)-2-octenal, 1-octen-3-ol and (E)-2-octen-1-ol, were further analyzed for MS confirmation.

Standard alkane ranging from C8-C20 in C6 solution were identified under the same condition with rabbit fish samples to obtain their retention time for linear retention index (LRI) calculation (Figure 3-7 and table 3-3).

The estimated concentration (ng/g sample) of each compound were calculated in terms of cyclohexanol. A total of 26 identified compounds could be classified into classes based on their chemical structure and general properties. As shown in Table 3-4, the volatile classes included 7 alcohols, 6 aldehydes, 5 ketones, 6 hydrocarbons and 5 other compounds. Most of these identified compounds have already been reported in several fresh seafoods [16–20].

When perceived by smell, viscera of rabbit fish possessed the most unpleasant smell compared to stomach contents, skin, and muscle. This was confirmed by GC-MS analysis results, which showed that the highest number and total concentration of volatile compounds was observed in viscera, followed by stomach contents, skin and muscle.

Fresh fish meat generally contains low levels of volatile compounds with mild and delicate smell [21]. In this study, only 2 carbonyl compounds, including hexanal and 1-octen-3-ol, were observed in fresh rabbit fish muscle, accounting for 156.92 ± 14.16, 133.55 ± 33.00 ng/g, respectively. A 1-octen-3-ol is one of the common eight-carbon compounds that have been identified in most fish, shellfish and crustacean species. Although this compound individually characterizes by a mushroom-like smell, it appears to represent the distinct heavy, fishy and grassy smell when it contributes to the fresh fish. Hexanal has also been identified as a component frequently found in both fresh and storage food, which represents a fatty-green and grassy odor. Comparatively high level of hexanal in rabbit fish was reported in study of Miyasaki & Fujioka [21] as well. Due to the fact that hexanal and 1-octen-3-ol have comparatively low odor threshold (hexanal, 5 μg/L; 1-octen-3-ol 1.5 μg/L), the OAVs, which calculated from estimated concentration of these compounds, were extremely greater than 1, suggested the strong contribution to the overall of smell in rabbit fish meat.

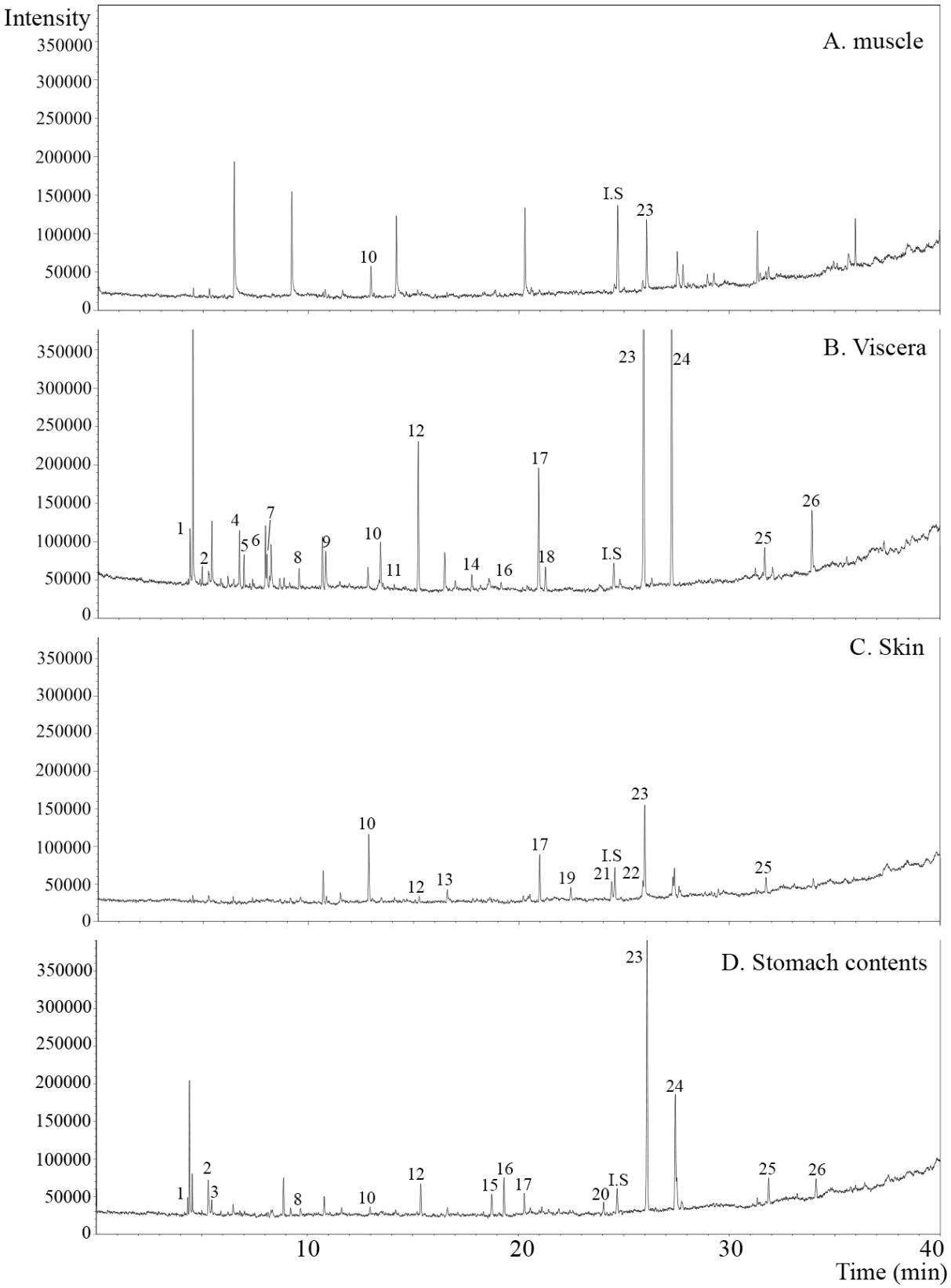

Figure 3-6 Chromatograms of volatile compounds obtained from rabbit fish tissues by SPME-GC-MS. Peak number are identified as in Table 3-2.

Table 3-2 Volatile compounds identified in headspace of rabbit fish tissues

Peak number	Retention time	LRI[A]	Compounds	Identification methods[B]
1	4.380	<600	Trimethylamine	MS, LRI, Std
2	5.284	758	Carbon disulfide	MS, LRI
3	5.430	769	Dimethyl sulfide	MS, LRI, Std
4	6.775	858	2-Octene	MS, LRI
5	6.976	871	(Z)-2-Octene	MS, LRI
6	8.021	924	(Z,Z)-3,5-Octadiene	MS, LRI
7	8.045	925	3-Methyl-butanal	MS, LRI, Std
8	9.633	987	3-Pentanone	MS, LRI
9	10.848	1031	1-Penten-3-one	MS, LRI, Std
10	12.889	1095	Hexanal	MS, LRI, Std
11	13.434	1111	(Z,E)-1,3,5-Octatriene	MS, LRI
12	15.263	1161	1-Penten-3-ol	MS, LRI, Std
13	16.625	1199	Heptanal	MS, LRI, Std
14	17.799	1231	Unknown, MW 122	
15	18.652	1254	1-Pentanol	MS, LRI
16	19.234	1269	3-Octanone	MS, LRI
17	20.982	1322	1-Octen-3-one	MS, LRI, Std
18	21.365	1335	(Z)-2-Penten-1-ol	MS, LRI, Std
19	22.525	1356	1-Hexanol	MS, LRI
20	24.005	1399	3-Octanol	MS, LRI
21	24.399	1410	Nonanal	MS, LRI, Std
I.S.	24.535	1414	Cyclohexanol	MS, LRI, Std
22	25.950	1453	(E)-2-Octenal	MS, LRI, Std
23	25.977	1454	1-Octen-3-ol	MS, LRI, Std
24	27.311	1492	3,5,5-trimethyl-2-Hexene	MS, LRI
25	31.685	>1600	(E)-2-Octen-1-ol	MS, LRI, Std
26	33.974	>1600	1-Dodecen-3-yne	MS, LRI

[A] Linear retention index on Supelcowax 10 capillary column.

[B] Definition: MS, identification by comparison of the fragment to the mass spectrum library (NIST11); LRI, identification by comparison with the LRI in the literature or in online standard reference database (https://webbook.nist.gov); Std, confirmation by authentic standards.

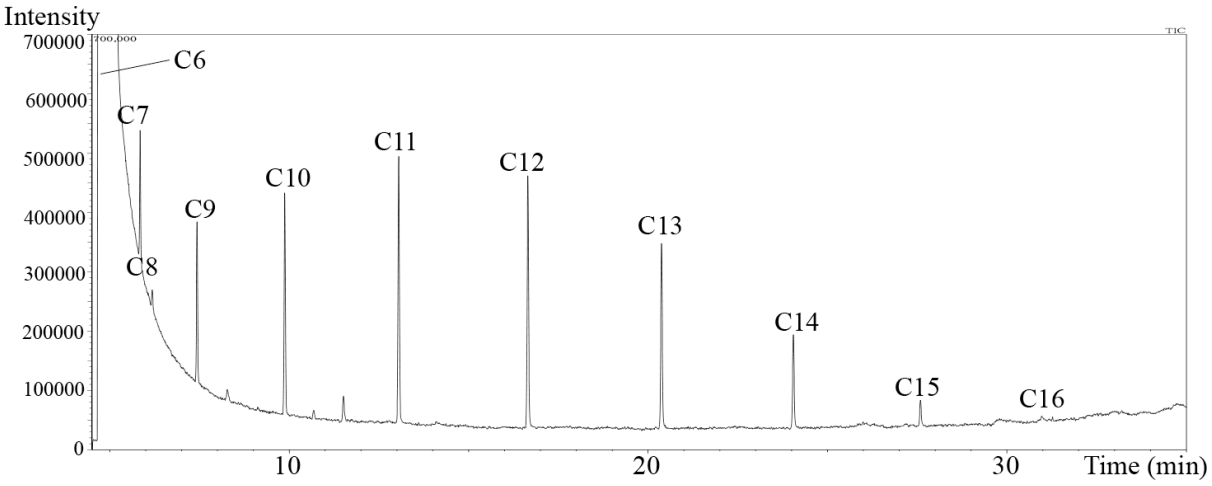

Figure 3-7 Chromatogram of standard alkanes solution. Peak number are identified as in Table 3-3.

Table 3-3 Volatile compounds identified in standard alkane solution

Peak number	Retention time	LRI	Compounds
C6	4.486	600	hexane
C7	4.970	700	heptane
C8	5.845	800	Octane
C9	7.430	900	Nonane
C10	9.875	1000	Decane
C11	13.050	1100	Undecane
C12	16.650	1200	Dodecane
C13	20.375	1300	Tridecane
C14	24.050	1400	Tetradecane
C15	27.590	1500	Pentadecane
C16	30.970	1600	Hexadecane

Viscera, skin and stomach contents of rabbit fish showed a higher number of volatile compounds than muscle. Alcohols contributed to the major volatile class, accounting for 43.1%, 53.9% and 52% of total volatile compounds, respectively. Three branched-chain alcohols, including 1-octen-3-ol, 1-penten-3-ol and (E)-2-octen-1-ol, were observed in all three tissues with relatively high level. However, considering the high threshold value of 1-penten-3-ol (358.1 μg/L), it might not contribute to overall smell of rabbit fish tissues, while another 2 compounds with a lower odor threshold might to.

Aldehydes were the second largest compounds in skin, accounting for 35.9% of total volatile compounds, but their levels were comparatively low in viscera (3.4%) and stomach contents (0.6%). Due to their low threshold values, aldehydes are important compounds in food, which could contribute to both desirable smell as well as unpleasant smell of spoilage and rancid fatty food [22]. A 3-methyl-butanal, which represented an almond and nutty smell, was observed at high OAV level in viscera. This compound might be generated by regular catabolic pathway of amino acids or from the Strecker degradation of amino acid [23, 24]. Hexanal that contributed to the overall smell of rabbit fish muscle also observed in viscera, skin and stomach contents.

Moderate level of ketones was observed in rabbit tissues (viscera, 12.3%; skin, 10.3%, stomach contents, 7.7%). However, because 1-octen-3-one, the major ketone compounds with a mushroom-like smell, has extremely low threshold value (0.005 μg/L) compared to other volatile compounds that was observed in rabbit fish tissues, which might result in the greater contributions to overall smell of rabbit fish than it corresponding alcohols (1-octen-3-ol). Although, 1-octent-3-one was not observed in fish muscle, the extremely high OAV level of this compounds in other three tissues may lead to the possibility that the over smell of the whole fish was associated with the presence of this compounds as well.

Hydrocarbons were abundantly observed in viscera. No odor threshold values were reported for hydrocarbons found in this study, but because hydrocarbons generally have high perception threshold, so they might not contribute to the overall smell in rabbit fish viscera.

Interestingly, volatile amines such as trimethylamine, which has been considered as the main compounds significantly represented the fishy and stale odor, were observed in viscera and stomach contents, but not in muscle of rabbit fish in this study. Trimethylamine is generated from microbial reduction of trimethylamine oxide, so it has been suggested as

indicators of spoilage, which could be used for the evaluation of fish freshness [25]. The absence of trimethylamine in rabbit fish meat in this study probably indicated that the fish meat was still fresh, and the unpleasant smell in rabbit fish meat did not occurred due to the microbial activity.

Fresh fish have distinguishing smell depending on the nature of the fish, which are often characterized by a species-related flavor. The major compounds commonly observed in fresh fish are several 6-, 8-, 9-carbons compounds [21], while the dominant volatile compounds in rabbit fish in this study were 5-, 6- and 8-carbons compounds. The formation of these carbonyls and alcohols compounds has been examined in several study, suggesting to be derived from long chain polyunsaturated fatty acids (PUFA) via the action of endogenous lipoxygenases [16], and could be classified as volatile lipid oxidation products [26]. Six and eight-carbons compounds, such as 1-octen-3-ol, 1-octen-3-one, (E)-2-octen-1-ol, (E)-2-octenal, are formed by the oxidation of n-6 PUFA, while 5-carbons compounds, such as 1-penten-3-ol and 1-penten-3-one are formed from n-3 PUFA [27, 28]. However, because different species of fish contain different types and concentration of lipoxygenase and PUFA, the type and concentration of volatile compounds might be also differently generated as well. Rabbit fish meats in this study contained high level of hexanal and 1-octen-3-ol, which probably formed by the oxidation of n-6 PUFA.

3.3.3 The formation of volatile compounds in rabbit fish meat during cold storage as fillets and as whole fish

Table 1-4 presents the change of volatile compounds in rabbit fish meat during cold storage as fillet and as whole fish. Total of 16 compounds were observed in rabbit fish meat during cold storage, including trimethylamine, 2-methyl butanal, 3-methyl butanal, 3-pentanone, hexanal, 1-penten-3-ol, heptanal, 3-methyl-1-butanol, 1-pentanol, 3-octanone, 2-penten-1-ol, 1-hexanol, nonanal, 1-octen-3-ol, benzaldehyde and 2-octen-1-ol. Significance increase (p<0.05) of total concentration (ng/ g sample) of volatile compounds in rabbit fish meats were observed after 4 days of storage as fillet, and after 2 days as whole fish storage. The levels of volatile lipid oxidation products from both n-3 and n-6 PUFA, such as hexanal, 1-octen-3-ol, 2-octen-1-ol and 1-penten-3-ol, were also significantly higher (p<0.05) in meat stored as whole fish than meat stored as fillet.

Moreover, trimethylamine, which is the compounds used for evaluation of fish freshness [25, 29], was also observed in the meat stored as whole fish after storage for 4 days, while in case of fillet storage, it could be observed after 6 days of storage. These indicated that rabbit fish stored as whole fish deteriorated more rapidly than stored as fillets, suggesting the importance of early evisceration on the smell quality of rabbit fish.

Viscera generally contains a spectrum of digestive enzymes that are able to hydrolyze protein, lipid and carbohydrate. These enzymes are engaged in autolysis of whole fish, which plays an important role in the fish spoilage [30]. After the death of fish, carbohydrase and lipase produce acidic pH, providing the optimum condition for protease activity. Therefore, proteases, a major enzyme observe in fish viscera, could hydrolyze and perforate the walls of digestive organs and leak out into surrounding tissues, which caused the general hydrolysis [31, 32]. Therefore, evisceration is the effective method to remove the gut enzyme.

Townley and Lanier [33] also reported that heading and evisceration of Atlantic croaker (*Micropogon undulatus*) and grey trout (*Cynoscion regalis*) soon after harvest could maintain the higher quality of fish meat during ice storage. Higher levels of trimethylamine were also observed in the whole herring (*Clupea harengus*) than in fillets, which reported to be probably due to the growth of TMAO-reducing bacteria because these bacteria prefer an anaerobic environment [34].

Moreover, results in this study suggested that a possibility that volatile compounds from fish viscera could migrate to another surrounding tissues, including the meat. The information about the migration of volatile compounds in fish tissues deserves further attention.

3.4 Conclusion

This chapter investigated the volatile compounds associated with the unpleasant smell in rabbit fish tissues, including muscle, viscera, skin and stomach contents, which are the basic information needed for further study about rabbit fish smell quality improvement. The key volatile compounds contributing to the overall smell of rabbit fish muscle were hexanal and 1-octen-3-ol, which could be classified as volatile lipid oxidation products (VLOP) from PUFA. Various types of carbonyls, alcohols, amines and sulfur-containing compounds were found in another 3 tissues. The highest level of OAV in these 3 tissues were found at 1-octen-3-one and 1-octen-3-ol, suggesting their strong association with the overall smell of whole fish. The generation of these volatile lipid oxidation compounds will be clarified and discussed further in chapter 5.

Furthermore, because various volatile compounds, including trimethylamine and VLOP, significantly higher in rabbit fish stored as whole fish than that of fillets fish, suggesting the importance of early evisceration on the smell quality of rabbit fish.

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Table 3-4 Estimated concentration[A] (ng/g) and OAVs (in parenthesis) of volatile compounds identified in rabbit fish *Siganus fuscescens* tissues

Compounds	Odor Description[B]	Odor threshold[C] (μg/L)	Estimated concentration (ng/g)							
			Muscle		Viscera		Skin		Stomach contents	
Alcohol (7)										
1-Penten-3-ol	Burnt, meaty	358.1	-		663.33 ± 136.04	(1.5)	28.25 ± 6.46	(0.1)	235.50 ± 59.07	(0.7)
1-Pentanol	Green, wax	150.2	-		-		-		215.21 ± 89.72	(1.4)
(Z)-2-Penten-1-ol	Green, plastic	89.2	-		111.03 ± 25.12	(1.2)	-		-	
1-Hexanol	Green, grassy	5.6	-		-		57.92 ± 16.00	(10.3)	-	
3-Octanol	Mushroom, herbaceous	35	-		-				60.50 ± 21.97	(1.7)
1-Octen-3-ol	Fishy, Grassy	1.5	133.55 ± 33.00	(89.0)	1877.43 ± 375.54	(970.4)	394.14 ± 81.62	(262.8)	1949.98 ± 464.50	(1300.0)
(E)-2-Octen-1-ol	Mushroom-like	50	-		158.15 ± 25.73	(2.4)	79.13 ± 9.14	(1.6)	218.56 ± 38.69	(4.4)
Subtotal			*133.55 ± 33.00*		*2809.94 ± 554.07*		*559.44 ± 101.14*		*2679.75 ± 484.83*	
Aldehyde (5)										
3-Methyl-butanal	Almond, nutty	1.1	-		86.79 ± 34.12	(96.7)	-		-	
Hexanal	Fishy, grassy	5	156.92 ± 14.16	(31.4)	105.31 ± 15.46	(20.1)	161.66 ± 67.80	(32.3)	34.60 ± 12.72	(6.9)
Heptanal	Dry fish	2.8	-		-		69.90 ± 16.42	(25.0)	-	
Nonanal	Citrus-like, soapy	3.4	-		-		112.34 ± 23.55	(33.0)	-	
(E)-2-Octenal	Oily, fishy	3	-		-		44.82 ± 19.52	(14.9)	-	
Subtotal			*156.92 ± 14.16*		*192.10 ± 43.45*		*417.57 ± 114.76*		*34.60 ± 12.72*	
Ketone (4)					-		-		-	
3-Pentanone	Irritant, acetone	316	-		80.45 ± 19.07	(0.3)	-		79.60 ± 42.71	(0.3)
1-Penten-3-one	Pungent, glue-like	1	-		194.26 ± 58.18	(148.3)	-		-	
3-Octanone	Herbal	21.4	-		-		-		149.23 ± 52.85	(7.0)
1-Octen-3-one	Mushroom-like	0.016	-		610.47 ± 207.77	(22494.0)	119.07 ± 48.15	(7442.1)	199.96 ± 171.58	(12497.4)
Subtotal					*885.18 ± 268.73*		*119.07 ± 48.15*		*428.79 ± 159.81*	

Table 3-4 (cont.) Estimated concentration[A] (ng/g) and OAVs (in parenthesis) of volatile compounds identified in rabbit fish *Siganus fuscescens* tissues

Compounds	Odor Description[B]	Odor threshold[C] (μg/L)	Estimated concentration (ng/g)							
			Muscle		Viscera		Skin		Stomach contents	
Hydrocarbon (6)									-	
2-Octene			-		185.27 ± 53.19		-		-	
(Z)-2-Octene			-		112.50 ± 25.78		-		-	
(Z,Z)-3,5-Octadiene			-		305.99 ± 113.81		-		-	
(Z,E)-1,3,5-Octatriene			-		196.42 ± 50.18		-		-	
3,5,5-Trimethyl-2-hexene			-		1353.30 ± 339.70		-		745.03 ± 140.36	
1-Dodecen-3-yne			-		322.60 ± 65.19		-		169.74 ± 33.44	
Subtotal			-		*2476.07 ± 482.27*		-		*914.76 ± 169.87*	
Others (4)										
Trimethylamine	Fishy, pungent	2.4	-		121.78 ± 27.20	(46.3)	-		729.44 ± 299.28	(303.9)
Carbon disulfide	Irritant	95.5	-		48.93 ± 26.76	(0.2)			180.27 ± 48.94	(1.9)
Dimethyl sulfide	Asparagus-like, putrid	0.84	-		-		-		105.81 ± 32.69	(126.0)
Unknown, MW 122 107, 122(40), 77(20), 108(7), 55(7), 65(5), 79(5)			-		88.19 ± 31.11		-		-	
Subtotal					*258.90 ± 53.251*		-		*1015.52 ± 296.77*	
Total			**290.46 ± 37.54**		**6622.19 ± 1294.00**		**1067.23 ± 226.29**		**5073.43 ± 608.92**	

[A] Estimated concentration of volatile compounds express as ng/g of sample. Data are means ± standard error (n = 4).
[B] Odor description and [C] odor threshold were obtained from the following literature [23, 28–32].

Table 3-5 Estimated concentration[a] (ng/g) of volatile compounds identified in rabbit fish meat during cold storage (4 °C) as fillet and as whole fish

Compounds	Day 0	Day 2		Day 4		Day 6	
		Fillet	Whole fish	Fillet	Whole fish	Fillet	Whole fish
trimethylamine	-	-	-	-	36.31 ± 6.43	254.33 ± 110.31	241.36 ± 19.77
2-methyl butanal	-	-	-	-	85.67 ± 38.58	29.41 ± 12.93	50.25 ± 5.34
3-methyl butanal	-	-	-	-	28.28 ± 10.73	23.20 ± 10.07	65.36 ± 11.06
3-pentanone	-	-	-	-	-	48.10 ± 9.51	29.12 ± 8.12
hexanal	110.64 ± 40.64 B	156.49 ± 31.34 B	562.26 ± 220.55 AB	147.43 ± 36.67 B	811.91 ± 303.23 A	198.75 ± 74.08 B	205.88 ± 52.85 B
1-penten-3-ol	-	24.74 ± 8.28 D	69.08 ± 14.76 CD	50.18 ± 20.80 CD	111.17 ± 22.29 BC	229.08 ± 68.31 AB	186.42 ± 18.63 A
heptanal	-	-	55.27 ± 13.32	40.50 ± 5.87	77.50 ± 15.07	40.48 ± 6.65	42.01 ± 13.13
3-methyl-1-butanol	-	-	-	-	-	539.97 ± 262.53	229.37 ± 61.46
1-pentanol	-	-	-	-	-	45.06 ± 16.35	25.80 ± 8.22
3-octanone	-	-	-	-	-	24.97 ± 8.77	28.51 ± 4.99
2-penten-1-ol	-	-	-	-	-	18.63 ± 7.30	17.33 ± 8.33
1-hexanol	-	-	-	-	40.81 ± 16.57	158.90 ± 43.57	79.23 ± 15.24
nonanal	-	27.20 ± 4.42	63.39 ± 19.07	38.50 ± 8.49	65.89 ± 4.81	43.49 ± 6.20	56.91 ± 7.95
1-octen-3-ol	-	202.36 ± 49.38 C	893.29 ± 255.24 A	271.47 ± 57.82 C	777.77 ± 130.25 AB	341.65 ± 117.32 BC	750.05 ± 66.13 AB
benzaldehyde	-	-	47.42 ± 33.59	-	179.68 ± 42.42	-	53.67 ± 18.11
2-octen-1-ol (z)	-	-	105.86 ± 27.86 A	-	122.38 ± 28.23 A	34.13 ± 7.05 B	75.45 ± 10.72 AB
Total	110.64 ± 40.64 B	410.79 ± 93.42 B	1741.29 ± 571.07 A	548.08 ± 129.65 B	2337.38 ± 618.60 A	2030.14 ± 760.94 A	2136.71 ± 330.06 A

[a] Estimated concentration of volatile compounds express as ng/g of sample. Data are means ± standard error (n = 4).

[A-D] Different letters indicate statistically significant differences ($p<0.05$).

Chapter 4 : Fatty acid composition in rabbit fish tissues

4.1 Introduction

It is generally accepted that polyunsaturated fatty acids (PUFA) have several beneficial effects on human health. Many studies have investigated the effect of PUFA, particularly the n-3 series such as EPA and DHA, on various health conditions. The data have indicated that these n-3 PUFA significantly diminish the incidence of cardiovascular diseases and sudden cardiac death [1–3] and suppress the increase of plasma cholesterol [4]. DHA is also essential for the formation of neuron synapses in the fetal brain [5], the improvement of learning ability and the development of visual function in infants [6–8].

In addition, several studies recently presented additional health benefits from other PUFA, such as n-6 PUFA ARA, which is presumed to also be essential for infant growth [9–11] due to its critical role in cell division and signaling [12], inflammatory response and immune system [13–15]. However, to satisfy their nutritional requirements, humans must obtain these PUFA from the diet because the synthesis of PUFA by chain elongation and desaturation of linolenic acid (18:2n-6) or alpha-linolenic acid in humans, especially in infants, is inefficient [16, 17]. Therefore, a natural and stable food source, which can provide the high levels of these fatty acids, is needed.

Marine fish are generally known as a major source of PUFA. Their lipids characteristically contain high levels of n-3 PUFA, especially DHA and EPA, which originate from phytoplankton through the marine food chain [18]. High levels of these DHA and EPA were reported in various marine fish species, such as mackerel scad (*Decapterus macarellus*) [19], sardine (*Clupea fimbriata*) [20], tuna (*Scomber australasicus*) [21], Caesioninae species (*Caesio diagramma*, C. tile), Siganidae species (*Siganus canaliculatus*) [22], and bonito (*Euthynnus pelamis*) [23]. On the other hand, only trace amounts of n-6 PUFA, such as AA,

are detected in marine fish [18]. However, there are several other marine species that contain comparatively high levels of n-6 PUFA in their lipids, including some kinds of algae [24, 25] and herbivorous species such as abalone [26] and oyster [27].

Recent studies have found that herbivorous fish also contain comparatively high levels of ARA [22, 28–30], and carnivorous fish that feed on sea urchins and sea snail, which consume microalgae, contain high levels of ARA as well as DHA [31]. Moreover, besides the high levels of DHA, surprisingly high levels of ARA have also been observed in top carnivorous predatory fish coral reefs, eating mainly small young and juvenile herbivorous fish such as Siganidae species [32]. This suggests that the high ARA levels might be the characteristic of herbivorous species, which may originate from ARA-rich macro-algae.

Rabbit fish, *Siganus fuscescens* (Houttuyn, 1782), is a tropical herbivorous fish species commonly found in Japan. This specie has been identified as one of the significant causes of diminishing seaweed beds that has occurred not only in Japanese coastal waters, but also in warm temperate waters throughout the world because of their high algae consumption [33–35]. The effective use of this herbivorous fish may be one method for recovering seaweed beds [36]. However, the utilization of this species has been limited due to the presence of unfavorable smell in its meat [36].

In general, due to the high level of PUFA, fish are very susceptible to oxidation, which leads to the formation of primary lipid oxidation products, including free radicals and hydroperoxides. The breakdown of these compounds to secondary products, such as aldehydes, ketones, and alcohols are considered to be responsible for the unpleasant odor in fish meat.

In chapter 3, the key volatile compounds that contributed to the smell of rabbit fish tissues were identified. The results indicated that the volatile compounds associated with the unpleasant smell in this species were the comparatively high levels of volatile lipids oxidation

products. Therefore, to understand the influence of lipids on the smell of rabbit fish, lipids and fatty acid composition in rabbit fish tissues were examined in this chapter, with a special focus on PUFA.

4.2 Materials and methods

4.2.1 Collection of fish specimens

Rabbit fish (average fork lengths 33.1±1.5 cm, average body weight 499.7±78.2 g, n=10) were caught off the coast of Nagasaki, Japan (32° 37'N, 128 ° 45'E) in September 9th, 2017 using set nets and transported to our laboratory under ice-storage condition. After arrival, the fish were immediately dissected into dorsal white muscle, dorsal skin, and viscera (including liver, intestine, kidney, mesenteric fat and gonad). Stomach contents were separated from viscera and also used as sample. All samples were frozen and kept individually at -80 °C until lipid extraction.

4.2.2 Lipid extraction

Rabbit fish tissues, including the dorsal ordinary muscles (white muscle), viscera, and skin, and their stomach contents were used as samples. Lipid was extracted by homogenizing sample with chloroform and methanol solution (2:1, v/v) according to the method of Folch et al. [38].

4.2.3 Fatty acid methyl esters preparation and gas chromatography (GC) analyses

The direct trans-esterification of the total lipids was carried out by boiling with methanol containing 1% of concentrated hydrochloric acid under reflux for 1.5 hr to produce the fatty acid methyl esters (FAME) [39]. Silica gel column chromatography was then used to

purify these methyl esters by eluting with chloroform/ether (10:1, v/v). FAME was determined using a gas chromatograph (GC-2014, Shimadzu Corporation, Kyoto, Japan) equipped with a 30m x 0.25mm x 0.25μm fused silica capillary column (Supelcowax^{TM}10, Supelco Japan Co., LTD, Tokyo, Japan). The split ratio was 10:1. The column was maintained at 205 °C, while the injection port and detector temperature were at 250 °C and 260 °C, respectively. Helium gas was used as carrier with a flow rate of 11.6 mL/min. Quantitation of individual components in lipids was expressed as percentage of total FAME based on peak areas.

4.2.4 Preparation of 4, 4-dimethyloxazoline derivatives and GC-MS analysis

To qualify the FAME, 4, 4-dimethyloxazoline derivatives (DMOX) were prepared by adding excess amount of 2-amino-2-methyl-propanol to small amount of FAME. The samples were heated at 180 °C for 18 hours and extracted with *n*-hexane [40]. DMOX were then purified using silica gel column by eluting with chloroform/ethanol (38:1, v/v) QP-2010 Ultra (Shimadzu Corporation, Kyoto, Japan) gas chromatograph mass spectrometer was operated to analysis the DMOX derivatives. The same capillary column as described above was used with 1:76 split ratio. The column temperature was at 205 °C, and the injector was maintained at 250 °C. MS spectra were recorded at 70 eV ionization energy. Helium gas was used as the carrier gas with a flow rate of 11.6 mL/min.

4.2.5 Identification of fatty acid methyl esters

Identification of fatty acid methyl esters was performed by (i) using marine lipid methyl esters as standards (Supelco 37 Component FAME Mix, Supelco Japan Ltd., Tokyo, Japan) and also by (ii) comparison of mass spectral data obtained from GC-MS.

4.2.6 Statistical analysis.

Data were analyzed using one-way ANOVA (IBM SPSS statistics, IBM, USA) and the differences between mean values were determined statistically with Duncan's New Multiple Range Test at $p<0.05$ (n=4).

4.3 Results & discussion

4.3.1 Lipid contents in tissues and stomach contents of rabbit fish

Total lipids contents in the tissues and stomach contents of rabbit fish are given in Table 4-1. Total lipid contents (% wet weight) in muscle, viscera, and skin were 0.34 ± 0.02%, 4.93 ± 0.51%, 1.86 ± 0.16%, respectively. Stomach contents contained pieces of seaweed, sand, and mucoid substances. The lipid contents in stomach contents was 1.74 ± 0.16%.

Low lipid levels have been observed in muscle of this specie [41], while the visceral lipid contents were significantly higher ($p<0.05$), suggesting that lipid deposition occurred mainly in the viscera, similar to that reported for other fish species [22, 32, 42, 43]. No significant association between the lipid contents in muscle and skin was observed of rabbit fish. Meanwhile, lipids in the stomach contents of rabbit fish was significantly present at low levels ($p<0.05$) compared to viscera, which suggest that they accumulate the lipids from their prey.

In this study, lipid contents of rabbit fish were comparatively lower than those observed in other studies [37, 44], which probably due to the seasonal variation. Rabbit fish tend to have the highest lipids levels in winter and low level during spawning period in summer [37]. Many marine fish species has been found to reduce their food intake during sexual maturation, and

presumed to use their stored lipids as an energy source for spawning and gonadal growth [45, 46].

4.3.2 Fatty acid composition in lipids of rabbit fish tissues

The fatty acid composition in total lipids of rabbit fish tissues (% total FAME) are shown in Tables 4-1. SFA was the main class of fatty acids in all tissues of rabbit fish, accounting for 39.34 ± 0.72%, 44.49 ± 0.24% and 40.40 ± 0.95% in muscle, viscera and skin, respectively, while MUFA and PUFA were present at moderate levels (MUFA, 24.05-29.36%; PUFA, 19.63-21.64%).

Although the lipid contents in viscera were significantly ($p<0.05$) greater, the major fatty acids observed in this tissue were similar to those in muscle and skin with slightly differed in the fatty acid composition ratios. The predominant fatty acid in all tissues was 16:0, followed by 18:1n-9 and 16:1n-7. DHA were found to be the dominant PUFA in all tissues, accounting for 7.12-7.50%, while ARA (3.04-4.28%) and DPA (2.94-4.25%) were observed in detectable levels in all tissues.

The most abundant fatty acid in the stomach contents of rabbit fish were SFA, accounting for 40.96 ± 0.41%, and the main fatty acids were 16:0, similar to those observed in rabbit fish tissues. However, significantly high level ($p<0.05$) of PUFA were observed in stomach contents of this fish. ARA and DHA were found to be the predominant PUFA, which also had significantly greater levels ($p<0.05$) than those in rabbit fish tissues.

In general, n-6 series of PUFA, such as ARA, are undetectable or present only in trace levels in marine fish lipids, whereas n-3 series, particularly DHA and EPA, which originate from phytoplankton through the marine food chain, are abundantly present [18]. High levels of

n-3 PUFA, especially DHA, have been reported in various marine fish [19–23]. This herbivorous fish assessed in this study also contained high levels of n-3 PUFA, especially DHA, in all tissues. These n-3 highly unsaturated fatty acids (HUFA) are generally known as essential for marine fish because of their important role in membrane fluidity [47]. They are also important for the normal development of embryos and larvae, as well as in the adaptation to changing environmental conditions [43, 47–49].

However, rabbit fish in this study contained not only n-3 PUFA but also markedly high levels of n-6 PUFA. The high n-6 PUFA levels, particularly ARA, are unusual. The lipid profile of this herbivorous species differed from other carnivorous and omnivorous marine species that consisted of mainly DHA and EPA [19–23, 50–53]. This might be due to the influence of diet, which is one of the main factors affecting the lipid contents and fatty acid profiles of marine organisms. In general, lipids profile of marine fish is typically affected by several interrelated external and internal factors, such as season, species habitat, age, size, sex, and life stage [23, 37, 54–59]. However, because marine fish are unable to synthesize some PUFA de novo [60], they must obtain these essential fatty acids from their diet.

The main food source of rabbit fish is brown algae, such as sargassum [34, 61–63]. These brown algae contain high levels of ARA [24, 25], similar to the lipids in tissues and stomach contents of this species in our study, which suggested that the high ARA levels in lipids of three herbivorous species originated from the consumption of those ARA-rich algae and directly influence the fatty acid composition of fish tissues.

Moreover, beside the high level of ARA, the variation in the other fatty acids compositions among the herbivorous species, especially in PUFA, in both tissues and stomach contents were reported [44]. Rabbit fish contained significantly higher level of DHA in its lipid than other two herbivorous species, including *C. japonicus* and *K. bigibbus*, while markedly

high level of EPA was reported in lipids of *C. japonicus*. The differences of these PUFA levels among these herbivorous species were probably due to the differences in their feeding behavior and pray compositions [34, 62–64]. Several juvenile and adult herbivorous species were found to consume some animal [65]. For instance, small amount of sessile animals and gelatinous zooplankton were observed in the stomach contents of adult rabbit fish [61, 62], but this behavior was not evident in *K. bigibbus* [64]. Our finding supported that feeding behavior and diet compositions influenced the fatty acid composition of these three herbivorous species.

Similar high ARA levels have been reported for other herbivorous fish and mollusks species that consume mainly algae, such as *Turbo cornutus* [26], *Crassostrea gigas*, [27], *Siganus canaliculatus* [22], *Siganus rivulatus* and *Siganus luridus* [28]. Moreover, surprisingly high ARA levels were also observed in Epinephelinae species, which are the predominant carnivorous predatory fish in coral reefs, eating mainly small young and juvenile herbivorous fish such as Siganidae species [32]. This suggested that the high ARA levels in herbivorous fish, which originate from the consumption of AA-rich algae could also accumulate in marine animals through the marine food chain, similar to that observed for n-3 PUFA [31, 32].

High levels of ARA have also been observed in many gonadal fish lipids [56, 66]. Moreover, ARA might also be an essential fatty acid for marine fish as well as n-3 HUFA due to their important role as the main eicosanoid precursor [67–69], which related to many physiological function, such as growth and survival [68], reproduction [70, 71], and environmental stress resistance [72] in various larval and juvenile marine fish species.

4.4 Conclusions

Rabbit fish contained high levels of PUFA, especially ARA, which differed from other carnivorous and omnivorous marine species that consisted of mainly DHA and EPA. From previous chapter, the key volatile compounds contributed to the overall smell in rabbit fish meats were hexanal and 1-octen-3-ol, which are suggested to derived from the oxidation of n-6 PUFA, such as ARA via lipoxygenase activity. Therefore, the unpleasant smell in rabbit fish tissues probably occurred by the oxidation of markedly high level of ARA in its tissues. The lipoxygenase generation of volatile compounds in rabbit fish tissues will be further discussed in next chapter.

Moreover, because rabbit fish contained high levels of ARA, as well as DHA and some other n-3 PUFA, which is proposed to have essential benefits for human health, especially with respect to infant growth [9, 10]. These fish also have potential applicability as sustainable ARA sources, such as for infant food.

4.5Cited literatures

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Table 4-1 The lipid contents (% wet weight) and the fatty acid composition[A] of total lipid in the respective organ of rabbit fish

	Muscle			Viscera			Skin			Stomach contents		
Lipid contents	**0.34**	**±**	**0.02b**	**4.93**	**±**	**0.51a**	**1.86**	**±**	**0.16b**	**1.74**	**±**	**0.16b**
Total SFA	**39.34**	**±**	**0.72b**	**44.49**	**±**	**0.24a**	**40.40**	**±**	**0.95ab**	**40.96**	**±**	**0.41ab**
12:0	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
14:0	4.01	±	0.10	6.31	±	0.32	4.52	±	0.05	3.84	±	0.11
15:0	0.69	±	0.02	1.12	±	0.11	0.78	±	0.03	0.88	±	0.05
16:0	28.61	±	0.72	28.77	±	0.25	28.86	±	1.02	25.15	±	0.52
17:0	0.68	±	0.03	1.10	±	0.04	0.76	±	0.05	0.87	±	0.03
18:0	5.35	±	0.19	7.19	±	0.13	5.47	±	0.19	10.23	±	0.17
Total MUFA	**29.32**	**±**	**0.42a**	**24.05**	**±**	**0.66b**	**29.36**	**±**	**0.63a**	**17.92**	**±**	**0.52c**
16:1n-7	8.80	±	0.55	7.92	±	0.21	9.54	±	0.48	4.24	±	0.11
18:1n-9	14.78	±	0.24	10.38	±	0.43	14.56	±	0.33	8.58	±	0.39
18:1n-7	4.80	±	0.13	5.08	±	0.30	4.26	±	0.06	4.26	±	0.16
20:1n-9	0.70	±	0.02	0.62	±	0.03	0.68	±	0.05	0.61	±	0.05
22:1n-11	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
22:1n-9	0.23	±	0.02	0.05	±	0.03	0.32	±	0.06	0.23	±	0.08
24:1n-9	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
Total PUFA	**21.64**	**±**	**0.45b**	**19.63**	**±**	**1.05b**	**20.33**	**±**	**0.86b**	**31.15**	**±**	**1.02a**
n-6 series	*8.67*	*±*	*0.44b*	*8.34*	*±*	*0.62b*	*7.47*	*±*	*0.35b*	*17.54*	*±*	*0.69a*
18:2n-6 (LA)	1.17	±	0.06a	0.99	±	0.06a	1.28	±	0.06a	1.32	±	0.09a
20:4n-6 (ARA)	4.28	±	0.41b	4.57	±	0.44b	3.04	±	0.22b	10.80	±	0.47a
22:4n-6	1.39	±	0.02	1.24	±	0.07	1.63	±	0.09	2.25	±	0.09
22:5n-6	1.83	±	0.05	1.53	±	0.08	1.53	±	0.09	3.16	±	0.10
n-3 series	*12.97*	*±*	*0.65a*	*11.29*	*±*	*0.53a*	*12.86*	*±*	*0.64a*	*13.62*	*±*	*0.58a*
18:4n-3	0.23	±	0.02	0.12	±	0.02	0.24	±	0.01	0.08	±	0.01
20:4n-3	0.42	±	0.02	0.26	±	0.01	0.45	±	0.03	0.23	±	0.02
20:5n-3 (EPA)	1.04	±	0.07a	0.58	±	0.08b	0.80	±	0.07ab	0.81	±	0.06ab
22:5n-3 (DPA)	3.78	±	0.25ab	2.94	±	0.11b	4.25	±	0.25a	2.77	±	0.13b
22:6n-3 (DHA)	7.50	±	0.33ab	7.39	±	0.39ab	7.12	±	0.33b	9.73	±	0.43a
Total	**90.30**	**±**	**0.37a**	**88.17**	**±**	**0.59a**	**90.09**	**±**	**0.57a**	**90.03**	**±**	**0.42a**

[A] Results are expressed as % of total FAME based on peak areas.

Data are mean ± standard error (n=4).

a-c Different letter in the row indicate statistical differences ($p<0.05$).

SFA, MUFA, PUFA means saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid, respectively.

ARA, EPA, DPA and DHA means arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid, respectively

Chapter 5 : Enzymatic generation of volatile compounds in rabbit fish tissues

5.1 Introdution

It is generally accepted that smell is one of the most important factors, which significantly impacts the consumer acceptance of fish. The smell of fresh fish varies among species, but it usually contains low level of volatile compounds with mild and delicate smell [1]. Many studies established that one of the key roles of the smell development in fish is lipoxygenase (LOX, EC 1.13.11.120). This enzyme is capable to catalyze the oxidation of PUFA to produce hydroperoxides, and lead to the formation of specific compounds, such as alkanes and aldehydes, which are responsible for the smell generation process [2, 3]. Josephson, Lindsay and Stuiber [4] firstly demonstrated that LOX is involved in the formation of carbonyls and alcohols, which present the specific fresh fish smells of emerald shiners *Notropis atherinoides*. The 8- and 9-carbon compounds that characterize the sweet smell of ayu *Plecoglossus altivelis*were also suggested to be generated from the oxidation of PUFA initiated by LOX in its skin [5].

From previous chapter, the volatile lipid oxidation products of n-6 PUFA, including hexanal and 1-octen-3-ol were identified as the key volatile compounds that contributed to the unpleasant smell in rabbit fish. Moreover, markedly high level of n-6 PUFA, especially ARA, was observed in rabbit fish tissues. These led to the possibility that rabbit fish may also contain LOX, which was capable to initiate the oxidation of PUFA and form the volatile compounds that associate with the unpleasant smell in its tissues. To examine the genesis of the unpleasant smell in rabbit fish, the oxidation of PUFA initiated by crude enzyme extracted from rabbit fish viscera were investigated in this chapter with a focusing on the generation of volatile products.

5.2 Materials and methods

5.2.1 Chemicals

Authentic reagents used for identification of volatile compounds were obtained from Tokyo Chemical Industries (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), Merck (Hohenbrunn, Germany) and Alfa aesar (Heysham, UK).

ARA, DHA and EPA were purchased from Nacalai tesque (Kyoto, Japan), while LA were from Wako Pure Chemical Industries (Osaka, Japan).

5.2.2 Fish sampling and preparation

Rabbit fish *Siganus fuscescens* (average fork lengths 33.1 ± 1.5 cm, average body weight 499.7 ± 78.2 g, n=10) were caught off the coast of Nagasaki, Japan (32° 37'N, 128° 45'E) using set nets and transported to our laboratory under ice-storage condition. After arrival, the fish was immediately dissected. Viscera was selected to be used for the preparation of crude enzyme for the lipid oxidation model due to their highest concentrations of volatile compounds generated from the reaction with ARA according to the preliminary experiment (Fig 5-1).

5.2.3 Crude enzyme extraction and PUFA substrate preparation

The crude enzyme was extracted following the method of Jin et al. [6] with slightly modification. The viscera of rabbit fish were homogenized with four volumes of cold 50 mmol/L sodium phosphate buffer (pH 7.4) containing 1 mmol/L dithiothreitol (DTT) and 1 mmol/L ethylenediaminetetra acetic acid (EDTA) at 10,000 r/min for 1 min in ice bath and centrifuged at 10,000 g, 4°C for 40 min. After filtrating through filter paper, the resulting

supernatant was collected and used as crude enzyme extract without further purification. Protein concentration of crude enzyme was determined according to the method of Lowry et al. [7]

The PUFA substrate solutions were prepared by individually mixing 10 mmol/L of commercial LA, ARA, DHA and EPA with 10 mmol/L sodium tetraborate buffer (pH 9.0) containing 0.1 mL/100 mL of Tween-20 [8].

5.2.4 Analysis of volatile compounds generated from lipid oxidation model system

Lipid oxidation model mixtures were prepared in a 20 mL headspace glass vial, by mixing 50 µl of crude enzyme with 250 µl of PUFA substrate solution in 2.7 ml of 50 mmol/L sodium phosphate buffer (pH 7.4). Control samples, which contained only 50 µl of crude enzyme or 250 µl of PUFA substrate solution in sodium phosphate buffer (pH 7.4) were also prepared. The vial was then flush with nitrogen gas and hermetically sealed with screw cap with silicone septa. The generation of volatile compounds in control and reaction mixtures were determined immediately without incubation and after 24 hours of incubation at 30 °C in the dark using SPME-GC-MS technique under the same condition described in section 3.2.4 Volatile compounds from four replicate samples were analyzed.

5.2.5 Identification and Quantification of volatile compounds

The volatile compounds were identified based on comparing and matching their mass spectra with those reported in the mass spectra libraries (NIST11). The identification was considered when at least 3 from 4 replications of sample containing a peak at same retention time with a higher than 85% similarity to the NIST mass spectrum. When available, some

authentic standards were analyzed for MS confirmation. Linear retention index (LRI) values of the compounds were determined following the method describe in section 3.2.5

Quantities of each volatile compounds was estimated by calculating the ratio of its peak area to the cyclohexanol internal standard peak area, which obtained from the total ion chromatograms. A response factors were all assumed to be 1. The estimated concentration of volatile compounds in lipid oxidation model system were express as ng/ml reaction mixture.

5.2.6 Statistical Analysis

Volatile compounds from four replicate samples were analyzed by one-way ANOVA. The differences between mean values were determined statistically with Duncan's New Multiple Range Test at $p<0.05$.

5.3 Results & discussion

5.3.1 Volatile compounds identified in control solution

Due to the strongest smell, and the highest concentration of volatile compounds observed among the reaction between ARA and enzyme extract from rabbit fish tissues, viscera were chosen for preparation of crude enzyme (Fig 5-1). Protein concentration of viscera extraction was 27.70 ± 2.86 mg/ml.

Figure 5-2 presents the chromatogram of volatile compounds obtained from crude enzyme extracted from viscera before and after incubation at 30 °C for 24 hours. The identification of volatile compounds was considered when at least 3 from 4 replications of sample containing a peak at same retention time with a higher than 85% similarity to the NIST

mass spectrum. All data obtained from volatile analysis in reaction mixture were identified regardless of the presence of contaminant components reported in chapter 3.

Headspace of crude enzyme solution that extracted from viscera contained a total of 6 identified compounds, including trimethylamine, 1-penten-3-one, 1-octen-3-one, nonanal and 1-octen-3-ol, which also were the major compounds observed in rabbit fish viscera in chapter 3. After incubation, several volatile compounds, including 2-pentylfuran, 3-octanone and hydrocarbons (octane, nonane, decane and undecane) were generated at comparatively low levels, while some carbonyls, including 1-penten-3-one, 1-octen-3-one and nonanal were undetectable.

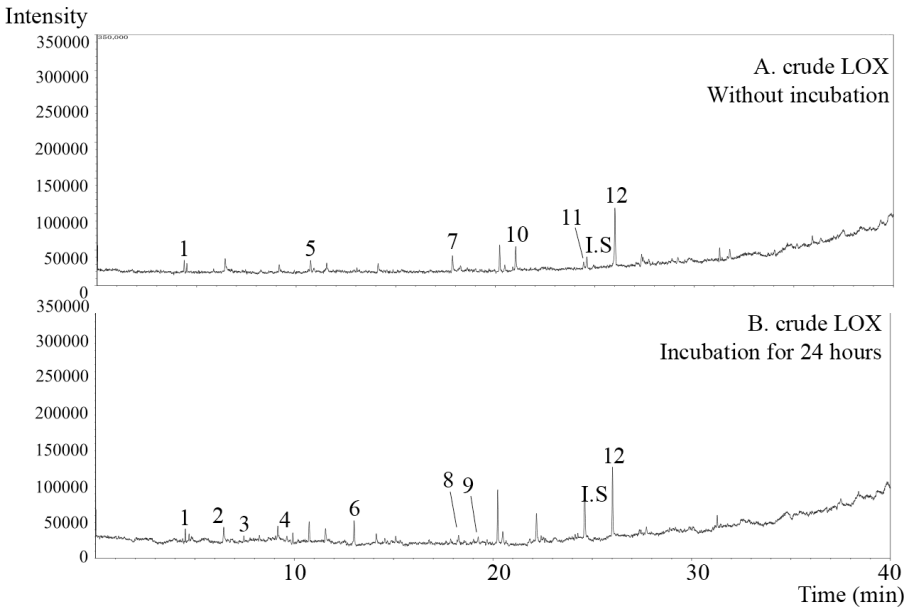

Figure 5-1 Chromatograms of volatile compounds obtained from crude LOX extract. Peak number are identified as in Table 5-1.

Table 5-1 Volatile compounds identified in enzyme solution

Peak number	Retention time	LRI	Compounds	Identification methods
1	4.380	<600	Trimethylamine	MS, RI, Std
2	5.854	800	Octane	MS, RI, Std
3	6.991	900	Nonane	MS, RI, Std
4	9.886	1000	Decane	MS, RI, Std
5	10.848	1031	1-Penten-3-one	MS, RI, Std
6	12.970	1100	Undecane	MS, RI, Std
7	17.799	1231	Unknown 1	
8	17.877	1243	2-Pentyl furan	MS, RI, Std
9	19.234	1269	3-Octanone	MS, RI
10	21.023	1317	1-Octen-3-one	MS, RI, Std
11	24.286	1410	Nonanal	MS, RI, Std
I.S	24.535	1414	Cyclohexanol	MS, RI, Std
12	25.963	1454	1-Octen-3-ol	MS, RI, Std

Four different PUFA solutions, including ARA, LA, DHA, and EPA, were used as substrates due to their comparatively high level in rabbit fish tissues. These 4 PUFA are also used as common substrates for determination of lipoxygenase activity. Figure 5-3 to 5-6 present the chromatogram of volatile compounds observed from the PUFA control solution of ARA, LA, DHA and EPA, respectively. Most of these compounds have been previously reports as products of the decomposition of n-3 and n-6 PUFA [9], and also usually found in fish and seafood, except for 2-heptanone (K1), 2-nonanone (K2) and 2-undecanone (K3), which contribute to fruity, floral and musty smell [10]. These odd-numbered ketones are unusually found in tissues of fish and other marine species, but they are commonly observed in dairy products, including milk and cheese [10]. In addition, 2-undecanone were reported to be the dominant compounds observed in fish oil [11] and fish oil-enriched mayonnaise [12]. The generation of these 3 compounds might be due to the β-oxidation of some fatty acids, or by the decomposition of β-keto acids [13]. Therefore, the analysis of volatile compounds in all reaction mixture were determined regardless of the presence of these 3 ketones components.

The concentration of volatile compounds observed in control sample were calculated and compared with those found in reaction mixture. Compounds in reaction mixture that had no significantly difference in concentration with the control solutions, were not considered as volatile oxidation products initiated by LOX.

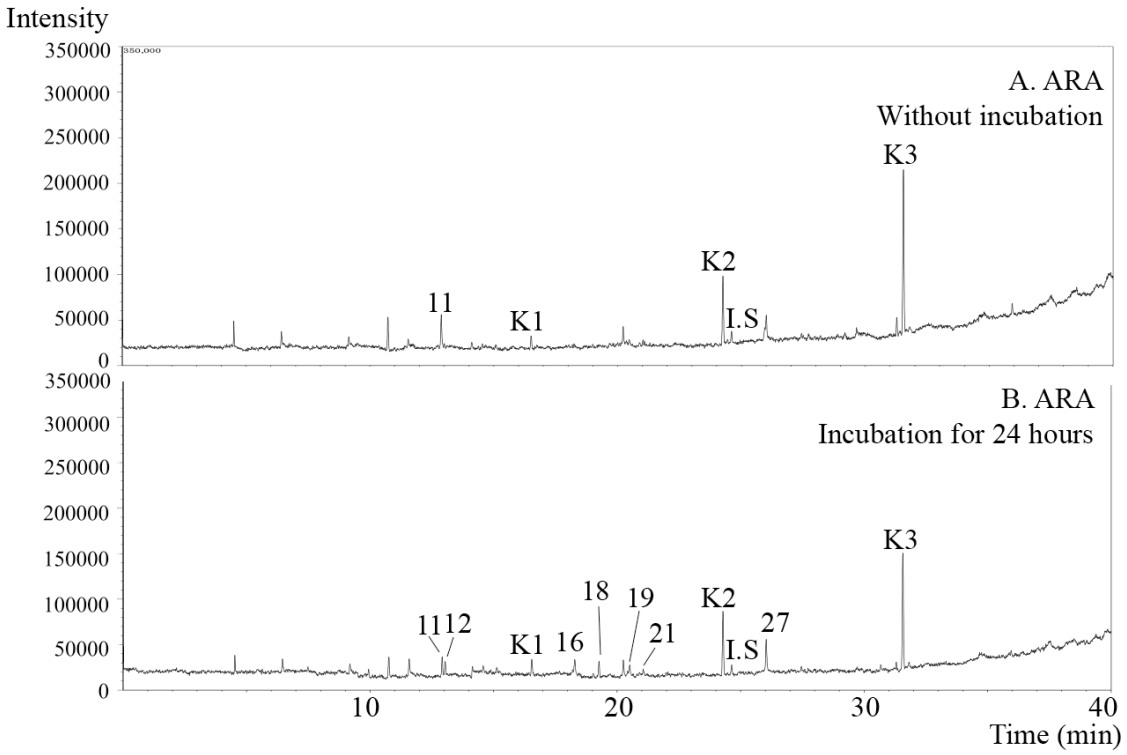

Figure 5-2 Chromatograms of volatile compounds obtained from ARA control solution. Peak number are identified as in Table 5-2.

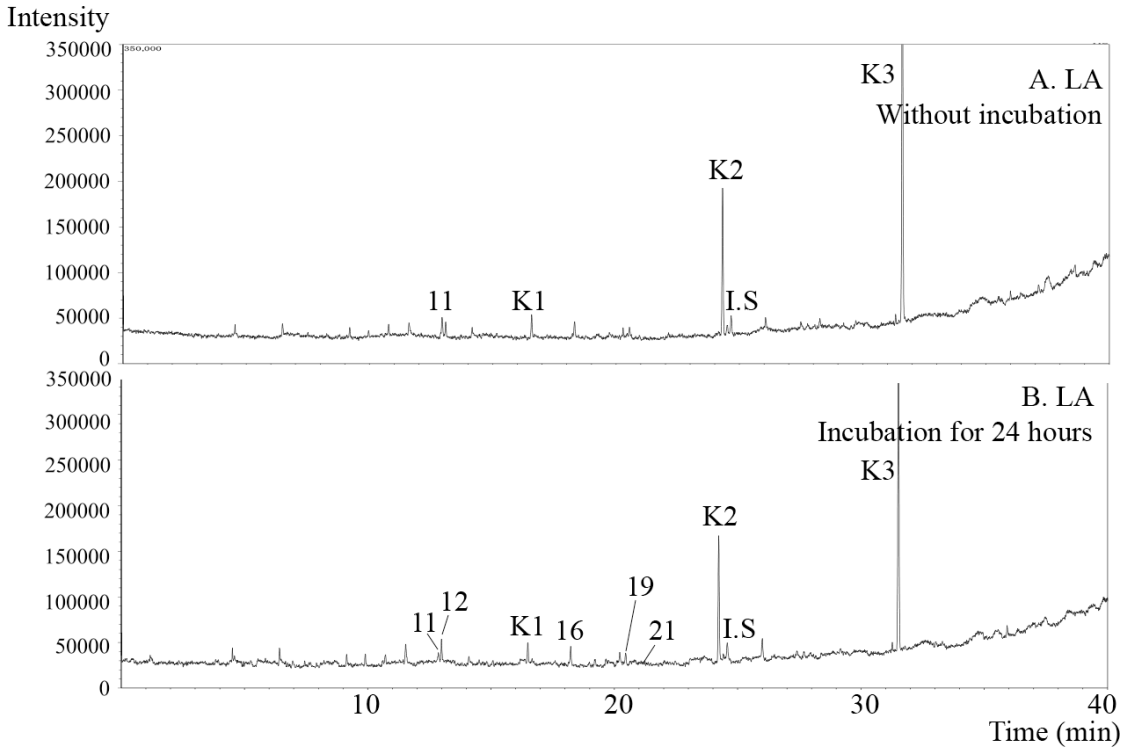

Figure 5-3 Chromatograms of volatile compounds obtained from LA control solution. Peak number are identified as in Table 5-2.

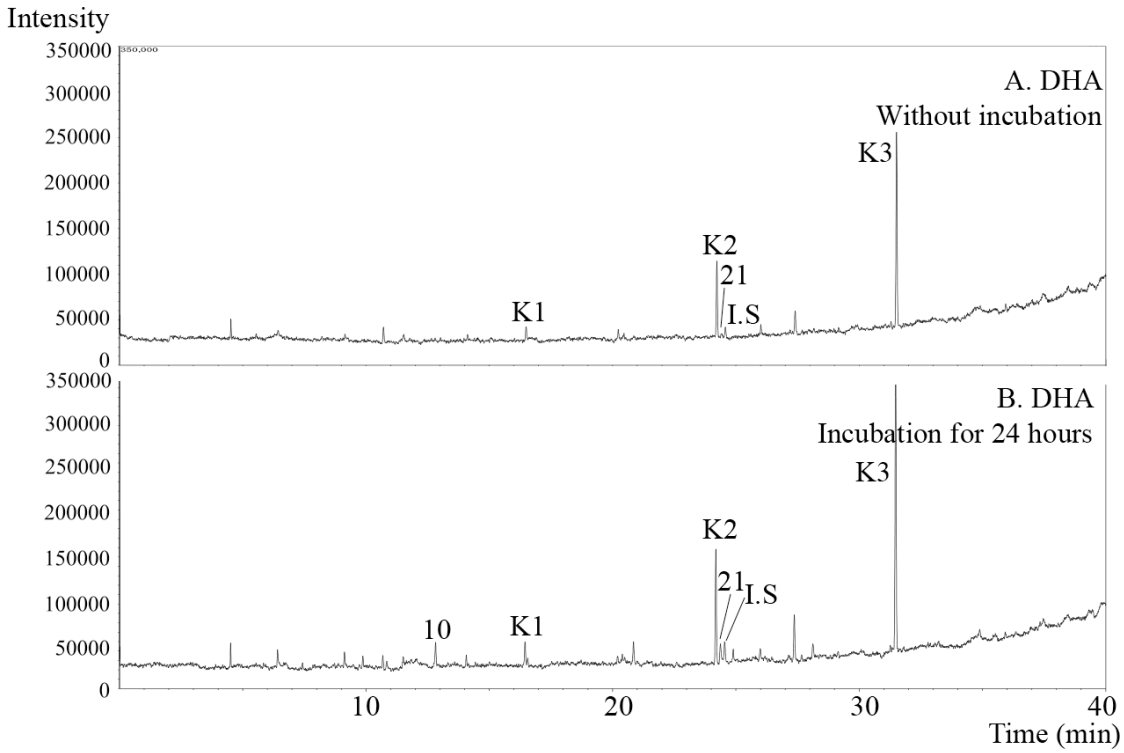

Figure 5-4 Chromatograms of volatile compounds obtained from DHA control solution. Peak number are identified as in Table 5-3.

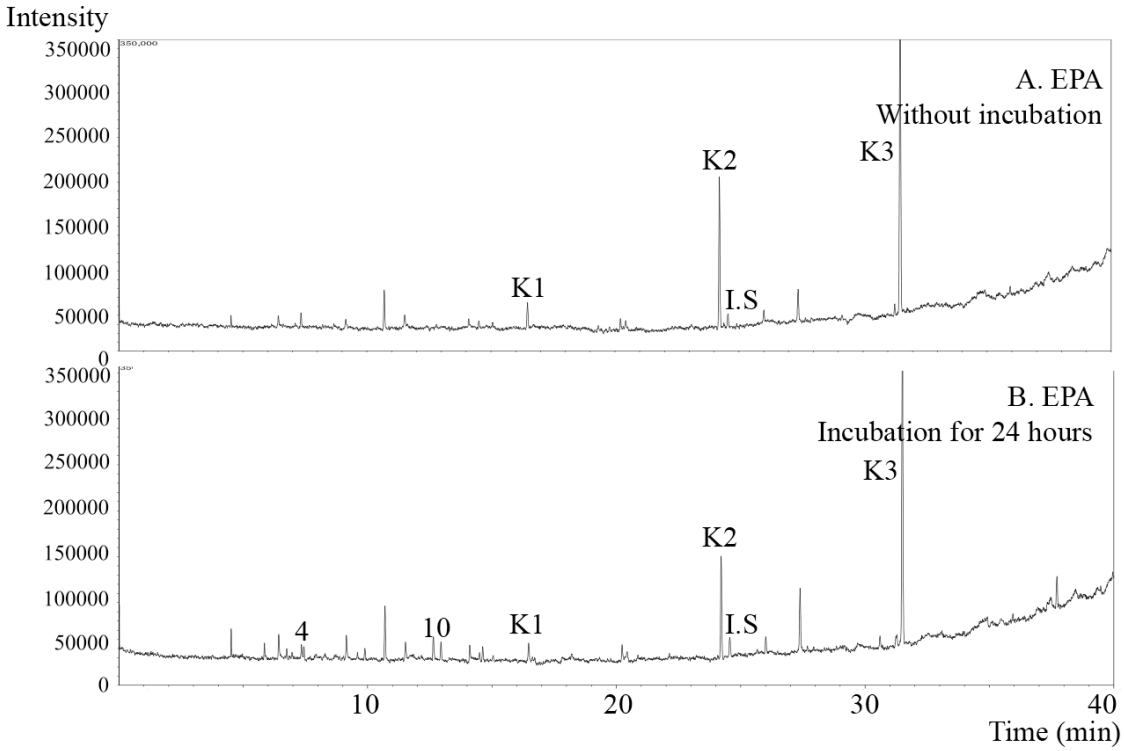

Figure 5-5 Chromatograms of volatile compounds obtained from DHA control solution. Peak number are identified as in Table 5-3.

5.3.2Volatile compounds generated from PUFA oxidation initiated by LOX

Figure 5-8 to figure 5-11 present the chromatogram of volatile compounds obtained from headspace of reaction mixture containing crude enzyme and ARA, LA, DHA and EPA, respectively. Peak number are identified and listed in table 5-2 (n-6 PUFA) and table 5-3 (n-3 PUFA). A total of 36 compounds were found in headspace of reaction mixture containing crude enzyme and n-6 PUFA, while 26 compounds were observed in those containing n-3 PUFA. Most of these have been already reported as the compound generated by LOX in various fish species, including emerald shiner [14], ayu skin [5] and silver carp meat [8]. This suggested that crude enzyme extracted from rabbit fish viscera contained LOX, which was able to catalyze the oxidation of PUFA and led to the generation of various volatile compounds.

Estimated concentrations of volatile compounds were calculated and expressed as ng per ml of reaction mixture, and presented in table 5-4 (LOX and n-6 PUFA) and table 5-5 (LOX and n-3 PUFA).

The activity of LOX in this study was shown as relative activity (%) calculated from the total amount of volatile compounds generated enzymatically from the breakdown of PUFA (Figure 5-12). Highest level of LOX activity ($p<0.05$) was clearly observed on ARA, follow by DHA and EPA, while LA showed the lowest level of LOX activity. This result was different from LOX extracted from other fish species. LOX extracted from sardine skin [15] and mackerel meat [16] showed highest reaction velocity on LA, while trout gill LOX extract showed the higher reaction on EPA than ARA or DHA [17].

For n-6 PUFA, reaction mixture of LOX and ARA shows the highest number of peak and highest concentration ($p<0.05$) of generated volatile compounds. The major volatile compounds in this reaction mixture were hexanal, 2-pentylfuran, and various of 8-carbon compounds, such as 1-octen-3-ol, 1-octen-3-one, 2-octen-1-ol, 2-octenal and 2-octene. Similar volatile compounds were also observed in reaction of crude enzyme and LA, though the levels of them were comparatively low. Eight-carbon compounds were reported as the products of 12-LOX on ARA as shown in chapter 2, figure 2-6 [18], while the act of 15-LOX give rise to six-carbons aldehydes such as hexanal from n-6 PUFA [19].

For n-3 PUFA, there was no significantly different between the total volatile compounds generated from reaction of crude enzyme containing DHA and EPA before incubation, though the reaction of EPA were significantly higher ($p<0.05$) than those of DHA after incubation. Some volatile compounds generated in the crude enzyme containing DHA and EPA in this study were also reported as the products of LOX, including (E,Z)-2,6-

nonadienal and 1-penten-3-ol. These 2 compounds were generated by the act of 12-LOX and 15-LOX, respectively (Chapter 2, Figure 2-7, [19]).

The presence of these volatile compounds suggested that crude enzyme from rabbit fish viscera probably contained mainly 12- and 15-LOX. These two type of LOX are also the major enzymes associated with the biogeneration of odor in various fresh fish [20].

Furthermore, the reaction between crude enzyme and PUFA in this study contained not only lipoxygenase-derived compounds, but also the compounds that could be generated only by lipid autoxidation as well. These compounds included (E,E)-2,4-decadienal, (E)-2-heptenal, (E,E)-2,4-heptadienal, (Z)-4-heptanal and (E,E)-3,5-octadien-2-one, which represent the rancid, stale, fishy off-odor in stored fish [21]. Due to their low threshold value, these compounds have significantly associated with the off-odor of fish and fish oil during storage. McGill et al. [22] presents the impact of (Z)-4-heptenal to the off-odor in cold stored cod. This compound has been reported to be generated from (E,Z)-2,6-nonadienal, which is mainly formed in LOX action [23]. Therefore, the presence of these autoxidation-derived compounds suggested that lipid autoxidation might be able to occur after the formation of fatty acid hydroperoxides that initiated by LOX.

Volatile compounds enzymatically generated from the breakdown of PUFA in this study were similar to the compounds that observed in rabbit fish tissues (chapter 3). The key odor compounds associated with the unpleasant smell in rabbit fish meat, including hexanal, 1-octen-3-ol, also the main volatile compounds generated from reaction of crude enzyme and ARA. The results suggested that the unpleasant smell of rabbit fish probably due to the presence of LOX in its tissues, which was capable to catalyze the oxidation of ARA, and led to the formation of volatile lipid oxidation products that contribute to the unpleasant smell.

In addition, unknown compounds with molecular weight of 122 and characteristic mass fragment; 107 (100), 122(40), 77(20), 108(7); Figure 5-6), were observed in reaction of crude enzyme and n-3 PUFA, in both DHA and EPA. The concentration of this compounds was highest among volatile compounds generated in this mixture, and also increased after incubation. This compound cannot be identified neither by MS library nor the comparation of LRI in other literature, and never be reported in the study of lipid oxidation, except for the study of Elmore et al. [24]. This compound was reported to be generated from lipid oxidation of α-linolenic methyl ester (18:3n-3) in meat-like model systems containing cysteine, ribose and PUFA.

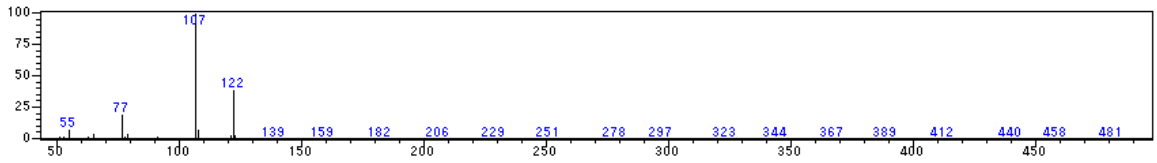

Figure 5-6 Mass fragments of unknown 1

5.4 Conclusion

The extraction of rabbit fish viscera probably contained LOX because various volatile compounds associated with the fresh fish smell, such as 5-, 6-, 8-carbon compounds were generated from the reaction with PUFA. These compounds were similar to the key odor compounds in rabbit fish tissues. Highest level of LOX activity ($p<0.05$) was clearly observed on ARA than EPA and DHA. The result suggested that the presence of the unpleasant smell in rabbit fish was probably due to LOX in its tissues, which was capable to catalyze the oxidation of PUFA, especially ARA, and led to the formation of volatile lipid oxidation products that contribute to the unpleasant smell of rabbit fish.

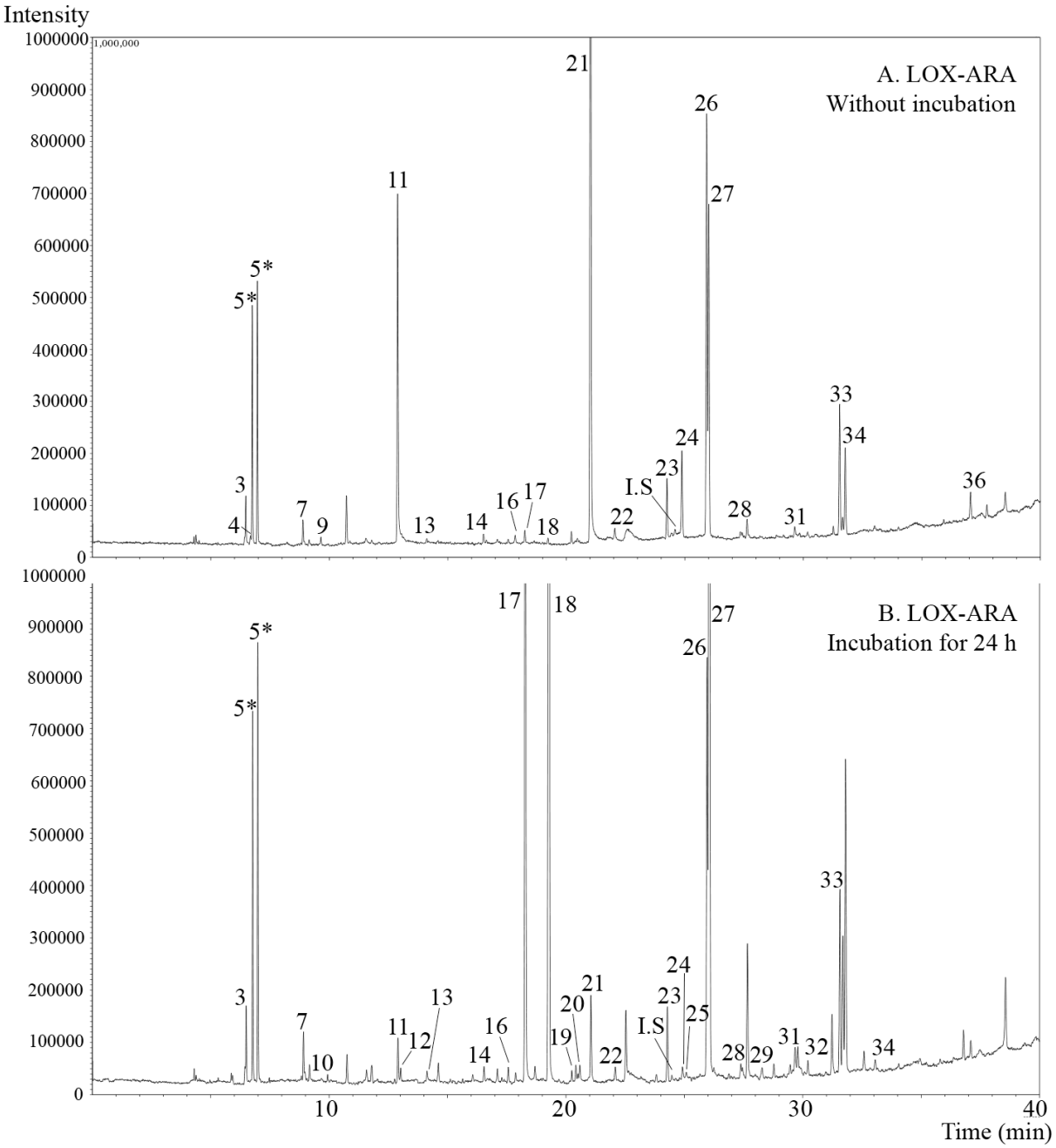

Figure 5-6 Chromatograms of volatile compounds obtained from reaction mixture containing LOX and ARA. Peak number are identified as in Table 5-2.

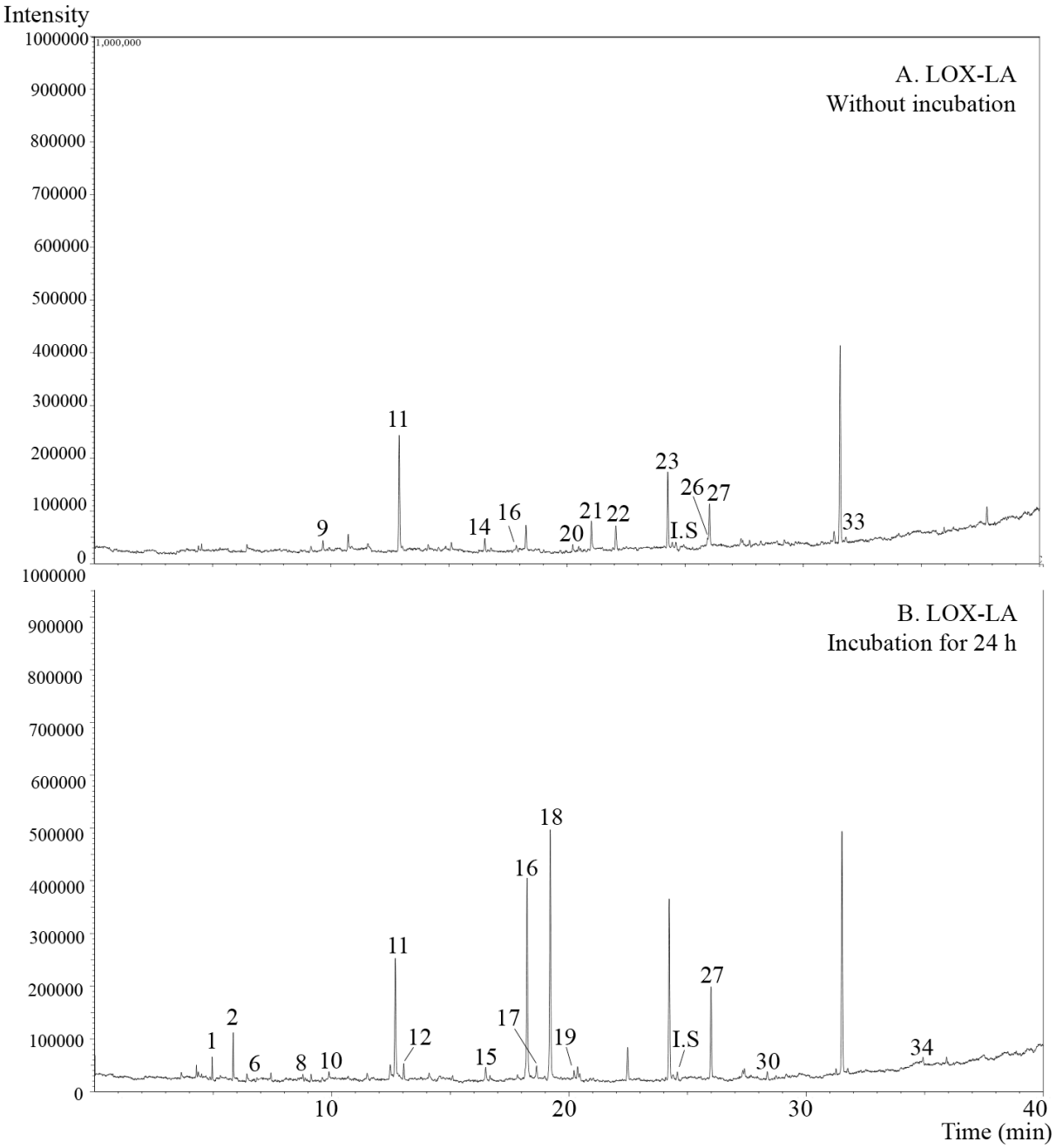

Figure 5-7 Chromatograms of volatile compounds obtained from reaction mixture containing LOX and LA. Peak number are identified as in Table 5-2.

Table 5-2 Volatile compounds identified in reaction mixture containing LOX and n-6 PUFA

Peak number	Retention time	LRI	Compounds	Identification methods
1	4.965	700	Heptane	MS, RI, Std
2	5.854	800	Octane	MS, RI, Std
3	6.499	840	1-Octene	MS, RI
4	6.670	852	2-Propenal	MS, RI
5	6.775	858	2-Octene*	MS, RI
6	6.991	900	Nonane	MS, RI
7	8.836	960	1,3-Octadiene	MS, RI
8	8.890	987	(E,E)-2,4-Heptadiene	MS, RI
9	9.650	991	Pentanal	MS, RI, Std
10	9.886	1000	Decane	MS, RI
11	12.767	1095	Hexanal	MS, RI, Std
12	12.911	1100	Undecane	MS, RI
13	14.135	1144	2-n-Butyl furan	MS, RI
14	16.630	1199	Heptanal	MS, RI, Std
15	16.658	1200	Dodecane	MS, RI
16	17.877	1243	2-Pentyl-furan	MS, RI, Std
17	18.286	1254	1-Pentanol	MS, RI, Std
18	19.234	1269	3-Octanone	MS, RI
19	20.252	1301	2-Octanone	MS, RI
20	20.513	1310	Octanal	MS, RI, Std
21	21.023	1317	1-Octen-3-one	MS, RI, Std
22	22.052	1346	(E)-2-Heptenal	MS, RI, Std
23	24.286	1410	Nonanal	MS, RI, Std
24	24.639	1424	1-Ethyl-1-methyl-cyclopentane	MS, RI
25	24.922	1429	3-Octen-2-one	MS, RI
26	25.084	1453	(E)-2-Octenal	MS, RI, Std
27	25.963	1454	1-Octen-3-ol	MS, RI, Std
28	27.454	1501	4-Pentyl-phenol	MS, RI
29	28.288	1535	trans-3-Nonen-2-one	MS, RI
30	28.367	1561	2-Nonanol	MS, RI
31	29.479	1561	(E)-2-Nonenal	MS, RI, Std
32	30.218	>1600	Cyclododecyne	MS, RI
33	31.570	>1600	(E)-2-Octen-1-ol	MS, RI, Std
34	33.056	>1600	(E,E)-2,4-Decadienal	MS, RI
35	34.933	>1600	2-Undecanol	MS, RI
36	37.749	>1600	Methyl dodecanoate	MS, RI
K1	16.567	1195	2-Heptanone	MS, RI
K2	24.307	1405	2-Nonanone	MS, RI
K3	31.591	>1600	2-Undecanone	MS, RI

* Compound observing at 2 retention time with similar m/z

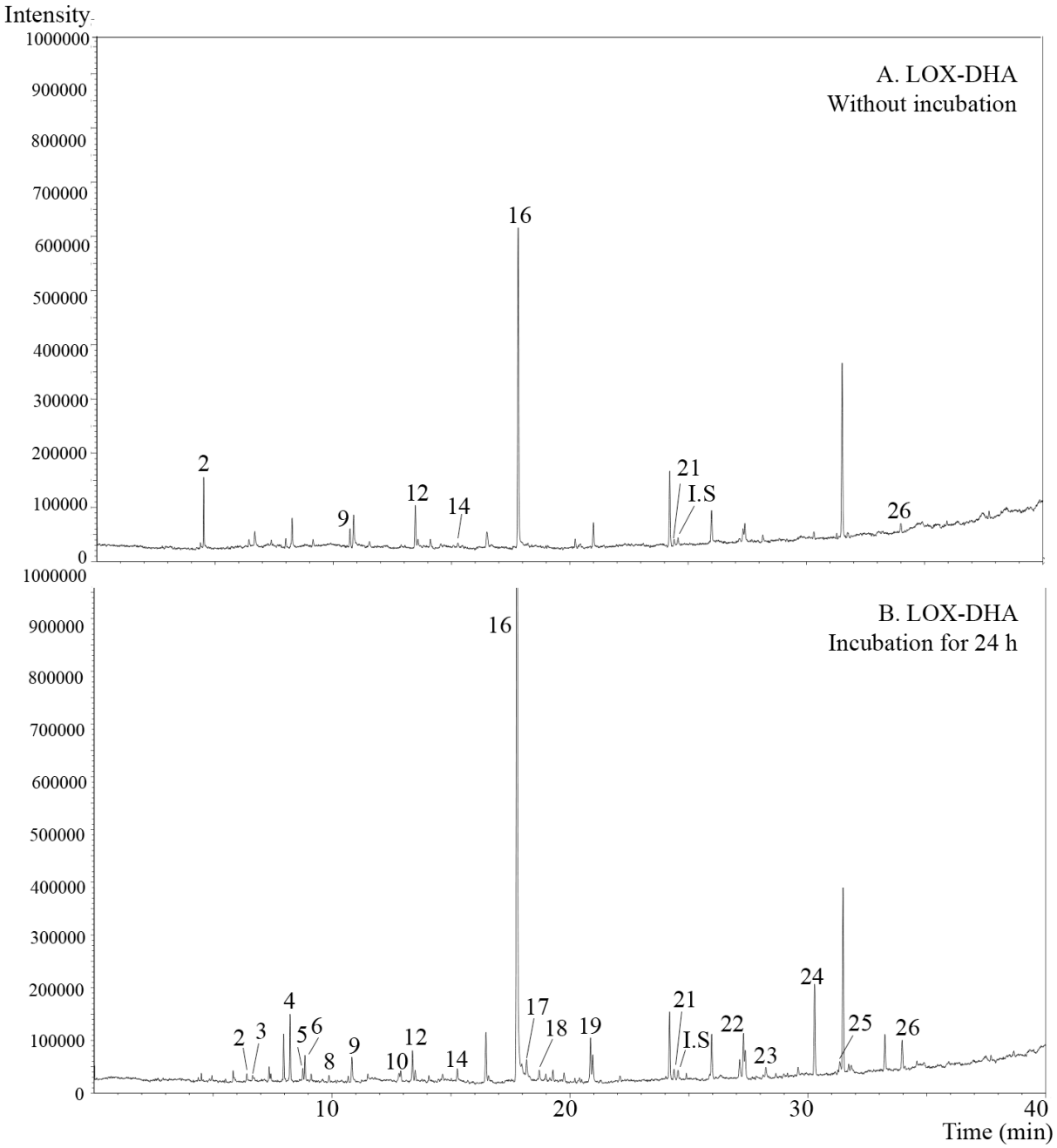

Figure 5-8 Chromatograms of volatile compounds obtained from reaction mixture containing LOX and DHA. Peak number are identified as in Table 5-3.

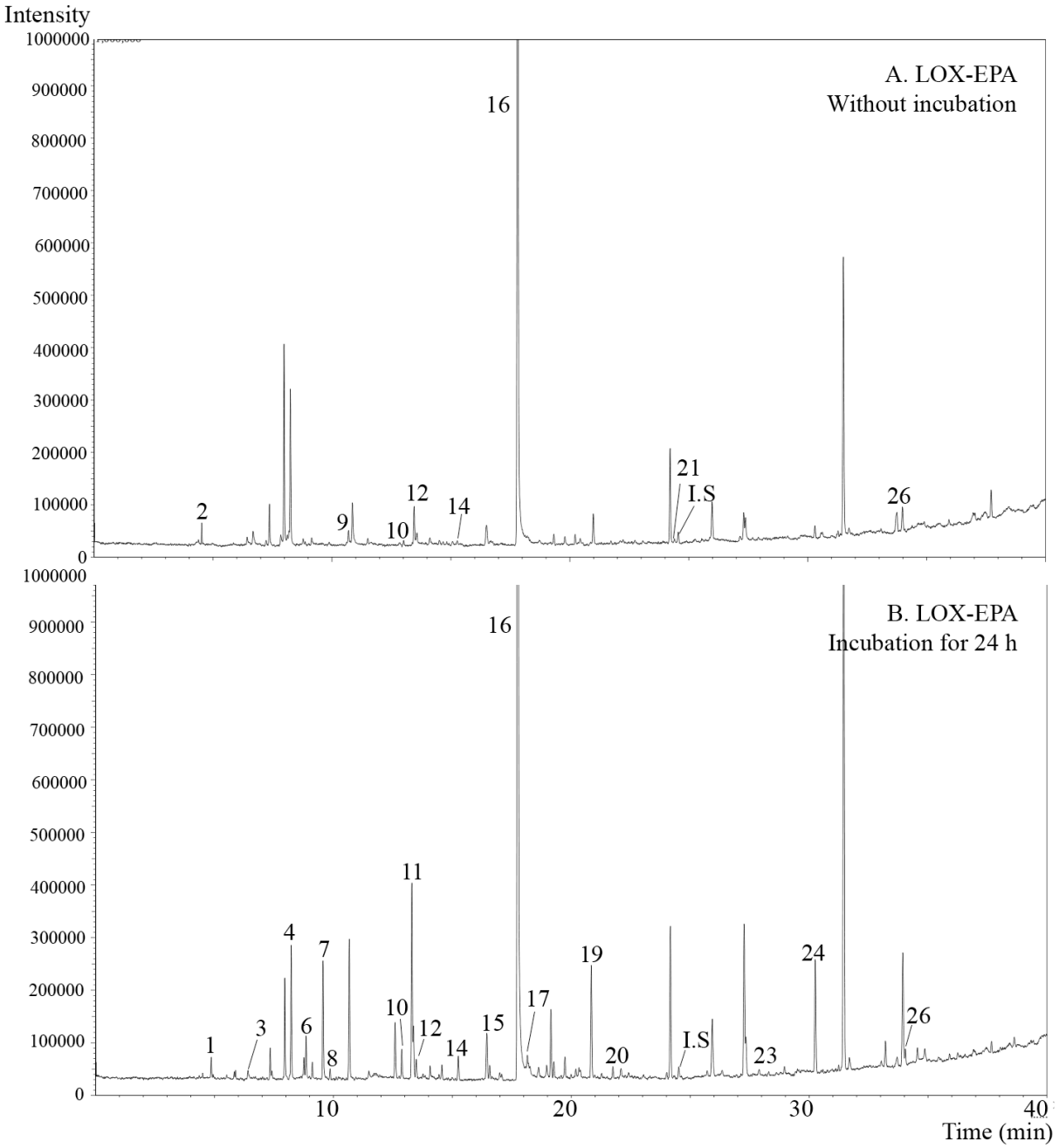

Figure 5-9 Chromatograms of volatile compounds obtained from reaction mixture containing LOX and EPA. Peak number are identified as in Table 5-3.

Table 5-3 Volatile compounds identified in reaction mixture containing LOX and n-3 PUFA

Peak number	Retention time	LRI	Compounds	Identification methods
1	5.854	800	Octane	MS, RI, Std
2	6.670	852	2-Propenal	MS, RI
3	6.991	900	Nonane	MS, RI, Std
4	8.180	931	(Z,Z)-3,5-Octadiene	MS, RI
5	8.785	955	2,2,4,6,6-Pentamethyl-heptane	MS, RI
6	8.876	959	2-Ethyl-furan	MS, RI, Std
7	9.633	990	3-Pentanone	MS, RI
8	9.886	1000	Decane	MS, RI
9	10.848	1031	1-Penten-3-one	MS, RI, Std
10	12.970	1100	Undecane	MS, RI
11	13.295	1112	Unknown	MS, RI
12	13.434	1111	1,3-trans,5-cis-Octatriene	
13	14.718	1146	(E)-2-Pentenal	MS, RI, Std
14	15.263	1161	1-Penten-3-ol	MS, RI, Std
15	16.650	1200	Dodecane	MS, RI
16	17.799	1231	Unknown, MW 122	
17	18.255	1243	2-Pentyl-furan	MS, RI, Std
18	18.712	1255	(Z)-4-Heptenal	MS, RI
19	20.854	1313	trans-2-(2-Pentenyl)furan	MS, RI
20	21.365	1327	(Z)-2-Penten-1-ol	MS, RI, Std
21	24.399	1410	Nonanal	MS, RI, Std
22	27.209	1489	(E,E)-2,4-Heptadienal	MS, RI
23	28.995	1542	(E,E)-3,5-Octadien-2-one	MS, RI
24	30.302	1580	Isosativene	MS, RI
25	31.358	>1600	(E,Z)-2,6-Nonadienal	MS, RI
26	33.974	>1600	1-Dodecen-3-yne	MS, RI
K1	16.567	1195	2-Heptanone	MS, RI
K2	24.307	1405	2-Nonanone	MS, RI
K3	31.591	>1600	2-Undecanone	MS, RI

Table 5-4 Estimated concentration[A] of volatile compounds identified in reaction mixture containing crude LOX and n-6 PUFA

Compounds	Odor description	Estimated concentration											
		No incubation						Incubation for 24 h					
		ARA			LA			ARA			LA		
Alcohols (5)													
1-Pentanol	Green, wax	39.10	±	3.26	-			186.57	±	54.47	83.33	±	30.82
1-Octen-3-ol	Fishy, grassy	4190.68	±	671.12	503.33	±	95.24	23876.53	±	5955.64	1000.09	±	39.31
2-Nonanol	Coconut, waxy, cucumber	-			-			-			95.01	±	3.84
(E)-2-Octen-1-ol	Muschroom-like	1283.07	±	273.85	92.87	±	13.85	3683.37	±	545.70	-		
2-Undecanol		-			-			-			301.63	±	123.75
Subtotal		*5512.85*	±	*933.14*	*596.20*	±	*91.01*	*27746.46*	±	*6537.28*	*1480.55*	±	*99.52*
Aldehydes (10)													
2-Propenal		101.30	±	17.04	-			-			-		
Pentanal	Pungent, almond like, rubber	108.37	±	15.15	145.64	±	19.02	-			-		
Hexanal	Fishy, grassy	4622.01	±	649.21	1722.65	±	197.78	2584.01	±	590.15	-		
Heptanal	Dry fish	63.72	±	1.57	59.45	±	8.06	174.25	±	16.80	-		
Octanal	Fatty, pungent	-			123.84	±	57.33	295.78	±	123.12	-		
(E)-2-Heptenal		203.38	±	27.94	451.02	±	67.65	242.64	±	47.33	-		
Nonanal	Citrus-like, soapy	63.44	±	10.06	115.91	±	22.62	118.34	±	10.64	-		
(E)-2-Octenal	Oily, fishy	5200.89	±	1031.90	149.47	±	9.69	6499.93	±	646.64	-		
(E)-2-Nonenal		176.09	±	25.29	-			668.61	±	151.80	-		
(E,E)-2,4-Decadienal	Fatty, deep-fried	645.78	±	121.42	-			901.46	±	230.19	-		
Subtotal		*11185.00*	±	*1855.09*	*2767.97*	±	*289.97*	*11485.04*	±	*1572.85*	-		
Ketones (5)													
3-Octanone	Herbal	106.61	±	10.49	-			14382.62	±	4102.79	2729.40	±	43.82
2-Octanone	Soapy, floral	-			-			192.15	±	57.32	238.03	±	52.16
1-Octen-3-one	Mushroom-like	15253.8	±	2515.31	621.80	±	156.81	4115.92	±	604.40	-		
3-Octen-2-one	Fatty, spicy	-			-			77.51	±	21.66	-		
trans-3-Nonen-2-one		-			-			43.48	±	20.80	-		
Subtotal		*15358.4*	±	*2522.22*	*621.80*	±	*156.81*	*18783.70*	±	*4358.92*	*2967.43*	±	*62.98*

Table 5-4 (cont.) Estimated concentration[A] of volatile compounds identified in reaction mixture containing crude LOX and n-6 PUFA

Compounds	Odor description	Estimated concentration											
		No incubation						Incubation for 24 h					
		ARA			LA			ARA			LA		
Hydrocarbons (12)													
Heptane			-			-			-		157.05	±	32.98
Octane			-			-			-		411.98	±	87.03
1-Octene		368.10	±	133.61		-		54.13	±	27.64		-	
2-Octene		3950.85	±	1296.75		-		601.55	±	198.93		-	
Nonane			-			-		39.06	±	5.70	89.21	±	13.48
1,3-Octadiene		234.09	±	54.84		-		119.05	±	31.18		-	
(E,E)-2,4-Heptadiene			-			-			-		39.31	±	14.70
Decane			-			-		68.31	±	11.93	94.71	±	8.18
Undecane			-			-		226.09	±	51.46	226.96	±	33.05
Dodecane			-			-			-		51.40	±	12.59
1-Ethyl-1-methyl-cyclopentane			-			-		136.88	±	27.89		-	
Cyclododecyne			-			-		518.57	±	185.64		-	
Subtotal		*5733.30*	±	*1510.10*		-		*1763.63*	±	*473.16*	*1070.63*	±	*125.00*
Others (4)													
2-n-Butyl furan	Noncharacteristics, weak	45.59	±	5.61		-		136.88	±	38.33		-	
2-Pentyl-furan	Green bean like	217.02	±	27.63	369.13	±	49.50	518.57	±	1123.64	1973.66	±	185.81
4-Pentyl-phenol		265.70	±	49.42		-		1763.63	±	80.81		-	
Methyl dodecanoate		194.30	±	37.83	271.04	±	22.92		-			-	
Subtotal		*722.60*	±	*90.67*	*640.17*	±	*69.37*	*8981.72*	±	*1171.96*	*1973.66*	±	*185.81*
Total		**38512.14**	±	**6286.76**	**4626.14**	±	**497.94**	**68760.55**	±	**12691.71**	**7492.28**	±	**178.48**

[A] Estimated concentration of volatile compounds express as ng in the headspace of 20 ml of reaction mixture. Data are means ± standard error (n = 4).

[B] Odor description was obtained from the following literature [25–30].

Table 5-5 Estimated concentration[A] of volatile compounds identified in reaction mixture containing crude LOX and n-3 PUFA

LRI	Compounds	Odor description	Estimated concentration			
			No incubation		Incubation for 24 h	
			DHA	EPA	DHA	EPA
	Alcohols					
1161	1-Penten-3-ol	Burnt, meaty	49.22 ± 6.43	38.18 ± 11.99	107.69 ± 13.34	169.78 ± 13.44
1327	(Z)-2-Penten-1-ol	Green, plastic	-	-	-	42.85 ± 6.53
	Subtotal		*49.22 ± 6.43*	*38.18 ± 11.99*	*107.69 ± 13.34*	*212.63 ± 19.53*
	Aldehydes					
852	2-Propenal		216.38 ± 23.77	103.13 ± 21.88	74.45 ± 7.83	-
1146	(E)-2-Pentenal	Green	-	-	90.95 ± 8.81	-
1255	(Z)-4-Heptenal	Biscuit, creamy, fatty	-	-	116.26 ± 8.06	-
1410	Nonanal	Citrus-like, soapy	95.81 ± 16.50	55.65 ± 8.18	104.61 ± 6.13	-
1489	(E,E)-2,4-Heptadienal	Fatty, hay, fishy odor	-	-	250.49 ± 69.91	-
>1600	(E,Z)-2,6-Nonadienal	Cucumber, wax, green	-	-	203.69 ± 21.27	-
	Subtotal		*312.19 ± 22.64*	*55.65 ± 8.18*	*844.44 ± 57.87*	-
	Ketones					
990	3-Pentanone		-	-	-	762.53 ± 149.44
1031	1-Penten-3-one	Pungent, glue-like	420.12 ± 51.16	309.04 ± 74.67	307.71 ± 20.03	-
1542	(E,E)-3,5-Octadien-2-one	Woody, mushroom, hay, fresh	-	-	47.68 ± 10.61	50.17 ± 19.29
	Subtotal		*420.12 ± 51.16*	*309.04 ± 74.67*	*355.38 ± 14.49*	*812.70 ± 146.69*

Table 5-5 (cont.) Estimated concentration[A] of volatile compounds identified in reaction mixture containing crude LOX and n-3 PUFA

Compounds	Odor description	Estimated concentration (ng/g)			
		No incubation		Incubation for 24 h	
		DHA	EPA	DHA	EPA
Hydrocarbons					
Octane		-	-	-	47.38 ± 13.90
Nonane		-	-	31.36 ± 13.21	4.42 ± 7.97
(Z,Z)-3,5-Octadiene		-	-	68.56 ± 26.13	1932.96 ± 449.44
2,2,4,6,6-Pentamethyl-heptane		-	-	94.52 ± 39.21	-
Decane		-	-	45.88 ± 12.29	59.33 ± 5.26
Undecane		-	64.79 ± 12.32	103.31 ± 13.47	143.46 ± 18.00
1,3-trans,5-cis-Octatriene		516.50 ± 50.93	386.86 ± 73.21	387.39 ± 54.84	429.24 ± 90.27
Dodecane		-	-	-	79.44 ± 13.42
1-Dodecen-3-yne		152.79 ± 40.12	200.29 ± 39.60	347.90 ± 24.93	792.47 ± 125.55
Subtotal		*669.28 ± 86.51*	*651.94 ± 102.93*	*1078.93 ± 53.52*	*3527.70 ± 647.08*
Others					
2-Ethyl-furan		-	-	299.97 ± 61.11	247.11 ± 29.59
Unknown 1, MW 122		5236.33 ± 424.63	6537.57 ± 1822.05	12563.74 ± 1054.19	19126.97 ± 2576.28
2-Pentyl-furan	Green bean like	-	-	179.06 ± 31.59	145.07 ± 20.78
trans-2-(2-Pentenyl)furan	Beany, grassy, buttery	-	-	673.29 ± 93.60	635.05 ± 68.48
Isosativene		-	-	752.43 ± 102.80	832.95 ± 176.61
Subtotal		*5236.30 ± 424.60*	*6537.57 ± 1822.05*	*14471.00 ± 1012.00*	*22069.70 ± 3147.50*
Total		**6687.10 ± 344.60**	**7695.52 ± 1968.20**	**16857.44 ± 1115.70**	**26622.80 ± 3914.20**

[A] Estimated concentration of volatile compounds express as ng in the headspace of 20 ml of reaction mixture. Data are means ± standard error (= 4).

[B] Odor description was obtained from the following literature [25–30].

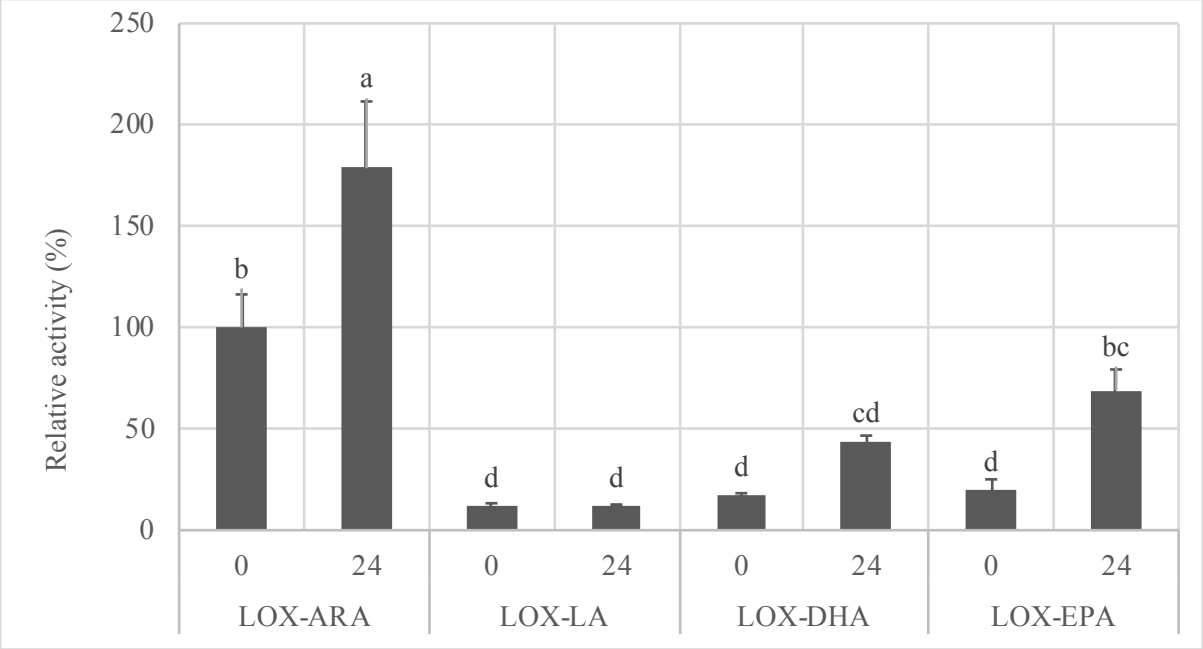

Figure 5-10 Relative activity (%) of crude LOX extracted from rabbit fish viscera. Different letters indicate statistically significant differences (p<0.05).

5.6 Cited literature

1. Lindsay RC (1990) Fish flavors. *Food Rev Int* 6:437–455.

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Chapter 6 : Effect of washing on the change of volatile compounds in rabbit fish meat during cold storage

6.1 Introduction

Rabbit fish (*Siganus fuscescens*) is a common herbivorous marine fish widely distributed in Japanese waters, but due to the unpleasant smell in its meat, the utilization of this fish as food source in Japan is limited. Therefore, this species has become overpopulation and consumed macroalgae excessively, which leads to be one of the significant causes involved in the reduction of seaweed beds that seriously occurred not only in coastal areas of Japan, but also in warm temperate waters around the world [1–3]. The effective utilization of this fish probably become one of the sustainable managements for restoring seaweed beds [4].

From previous chapter, volatile compounds associated with the unpleasant smell in rabbit fish were lipid oxidation products (VLOP), such as hexanal, 1-octen-3-ol, 1-octen-3-one, 1-penten-3-ol, and 1-penten-3-one. These various compounds were formed due to the oxidation of n-3 and n-6 PUFA, which were observed at high levels in its meat, and were catalyzed by lipoxygenase (LOX) in its tissues. Therefore, in order to remove the unpleasant smell in rabbit fish meat, the method that could prevent the lipid oxidation or decrease the level of VLOP is needed.

In the production of fish protein gel, such as surimi products, the essential step used for the gel quality improvement is washing. This step could remove not only the impurities in fish meat, but also the water-soluble protein, mainly sarcoplasmic protein including heme protein and various metabolic enzymes that impede the gel-forming ability of surimi [5]. Recently, washing step has been reported to have an impact on lipid oxidation of washed horse mackerel (*Trachurus trachurus*) meat, resulting in the less formation of VLOP, such as 1-penten-3-ol

and 2,4-heptadienal, during cold storage [6]. Moreover, this step also affected the overall smell of washed silver carp (*Hypophthalmichthys molitrix*) meat by washing away the off-flavor or facilitating the release of other volatile compounds [7]. Furthermore, adding antioxidants either in grinding step or washing solution has successfully prevented the oxidation in fatty fish, such as Atlantic mackerel (*Scomber scombrus*) [8, 9] and horse mackerel [10], resulting in the lower level of VLOP, including hexanal and 1-penten-3-ol, in meat washing with antioxidants solution than that in meat washing with water during storage [10].

Therefore, in the last chapter, the effect of washing on the quality of rabbit fish meat was investigated with special focus on the volatile compounds. Sodium ascorbate was used as an antioxidant washing solution. The formation of volatile compounds in both water and ascorbate washed meat during cold storage was observed using SPME-GC-MS.

6.2 Materials and methods

6.2.1 Fish sampling and preparation

Rabbit fish *Siganus fuscescens* (fork lengths 31.9 ± 1.7 cm, body weight 582.5 ± 73.8 g, n=10) were caught off the coast of Nagasaki, Japan (32° 37'N, 128° 45'E) using set nets in May 2018 and transported to our laboratory under ice-storage condition.

6.2.2 Fish washing and storage

Fish samples were dissected and eviscerated to obtain the filleted meat, then ground using a grinder. The washed meat was prepared following the methods of Takahashi *et al*. [11] with slightly modification. The obtained mince was washed by homogenizing (PT 10-35 GT; Kinematica, Lucerne, Switzerland) at 10,000 rpm, for 1 min in cold (4 °C) distilled water (W)

or 0.5% sodium ascorbate solution (A) at a ratio 1:3 (w/v). Samples were allowed to stand in ice bath for 10 min. Then, the mince was dewatered by centrifuging at 20,000 x*g* for 20 min at 2 °C. The method was repeated for three times. Four samples were obtained during the process, including unwashed meat after grinding (U), meat obtained from 1st wash (W1), meat obtained from 2nd wash (W2), and meat obtained from 3rd wash (W3). Color, pH, moisture contents and levels of volatile compounds, including hexanal, and 1-octen-3-ol were determined.

For the storage experiment, the meat obtained from 3rd wash from both water and ascorbate solution were kept in polyethylene bag and stored at 4 °C in the dark. Unwashed meat (U) was also used as control sample. Volatile compounds were analyzed after 2, 4, and 6 days of storage.

6.2.3 Volatile compounds analysis

Volatile compounds in sample were analyzed using SPME-GC-MS according to the method describe in chapter 3. Results were expressed as ng/g sample.

6.2.4Statistical Analysis

Moisture content, pH, color, volatile compounds contents, including hexanal, 1-octen-3-ol and 1-penten-3-ol, from four replicate samples were analyzed by one-way ANOVA. The differences between mean values were determined statistically with Duncan's New Multiple Range Test at $p<0.05$.

6.3 Results and discussion

6.3.1 Characterization of rabbit fish minces during washing

Table 6-1 shows the characterization of rabbit fish meat, including whiteness, color difference (ΔE), pH and moisture contents, during washing process with different washing times and washing solution.

Color of washed meats were significantly improved after washing by both water and ascorbate solution. The highest level of whiteness ($p<0.05$) was observed in the meat washed by ascorbate for three times. This could be explained by the removal of the impurities in fish meat and also red color protein, such as hemoglobin and myoglobin, during washing process [5]. For pH, washing with both solutions resulted in the significance increase of pH ($p<0.05$), which probably due to the removal of accumulated lactic acid in fish meat after their death by repeated washing process [12]. Moreover, although the pH of 0.5% sodium ascorbate solution (pH 7.37) was higher than that of cold water, there was no significance associated between pH of meat that washed by both solutions. Moisture content (%) increased during washing process with significantly higher ($p<0.05$) in the third times of water washed meat than ascorbate washed meat.

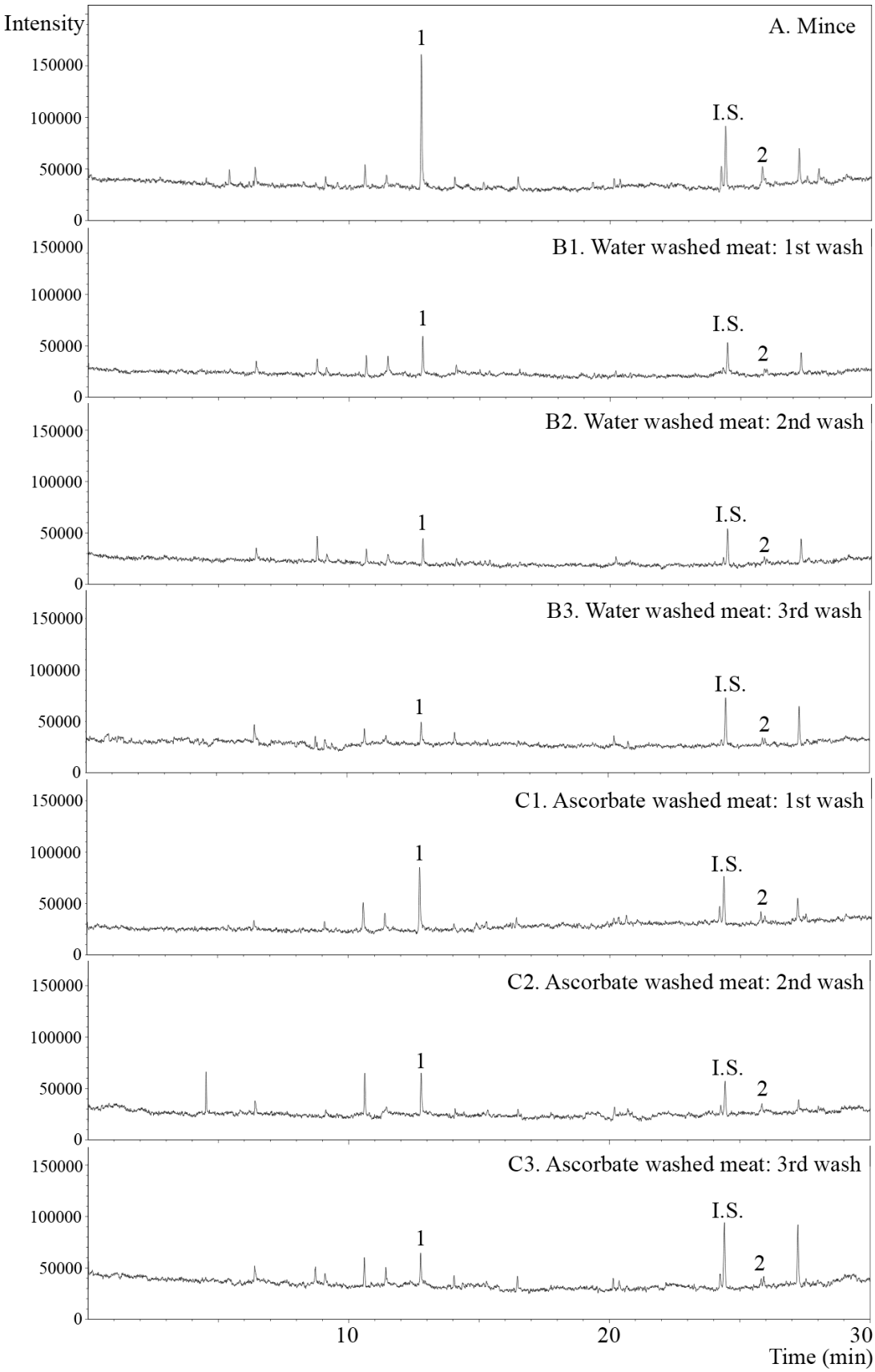

Figure 6-1 Chromatograms of volatile compounds obtained from rabbit fish meat during washing with different washing solution. Peak number are identified as in Table 6-2

Table 6-1 Characterization of rabbit fish minces obtained during washing with different washing solution

Sample	Color		pH	Moisture content (%)
	Whiteness	ΔE		
Unwashed meat	42.2 ± 2.4 d	-	6.70 ± 0.08 bc	80.00 ± 1.36 d
1st Water washed meat	56.7 ± 1.7 c	14.9 ± 1.9 c	6.82 ± 0.10 abc	85.65 ± 0.99 c
2nd Water washed meat	58.8 ± 2.8 c	17.4 ± 3.0 c	6.87 ± 0.06 ab	87.30 ± 1.70 bc
3rd Water washed meat	57.9 ± 1.2 c	16.2 ± 1.4 c	6.89 ± 0.09 a	90.55 ± 1.25 a
1st Ascorbate washed meat	59.7 ± 1.6 b	17.9 ± 1.3 b	6.68 ± 0.12 c	85.75 ± 1.29 c
2nd Ascorbate washed meat	60.4 ± 0.7 ab	18.5 ± 0.7 ab	6.79 ± 0.15 abc	85.88 ± 0.95 c
3rd Ascorbate washed meat	61.4 ± 0.9 a	19.4 ± 1.0 a	6.81 ± 0.14 abc	88.10 ± 1.20 b

Data are means ± standard errors (n=4).

a-d Different letter in the row indicate statistical differences ($p<0.05$).

Table 6-2 Volatile compounds identified in headspace of rabbit fish meat

Peak number	Retention time	LRI	Compounds	Identification methods
1	12.767	1095	Hexanal	MS, RI, Std
2	25.963	1454	1-Octen-3-ol	MS, RI, Std
3	15.263	1161	1-Penten-3-ol	MS, RI, Std
4	7.930	920	2-Methyl-butanal	MS, RI, Std
5	8.045	925	3-Methyl-butanal	MS, RI, Std
6	16.625	1999	Heptanal	MS, RI, Std
7	22.525	1359	1-Hexanol	MS, RI
8	4.370	<600	Trimethylamine	MS, RI, Std
9	9.633	987	3-Pentanone	MS, RI, Std
10	16.915	1207	3-Methyl-1-butanol	MS, RI
11	18.625	1254	1-Pentanol	MS, RI
12	19.234	1259	3-Octanone	MS, RI
I.S.	24.535	1414	Cyclohexanol	MS, RI, Std

[A] Linear retention index on Supelcowax 10 capillary column.

[B] Definition: MS, identification by comparison of the fragment to the mass spectrum library (NIST11); LRI, identification by comparison with the LRI in the literature or in online standard reference database (https://webbook.nist.gov); Std, confirmation by authentic standards.

Figure 6-1 shows the chromatogram of volatile compounds obtained from rabbit fish unwashed meat and meat after the 1st, 2nd and 3rd washing with different washing solution, including water and 0.5% sodium ascorbate. The change of volatile compounds in meat during washing process were presented by the concentration (ng/g) of hexanal (Fig 6-2) and 1-octen-3-ol (Fig 6-3) because they were the key odor compounds observed in rabbit fish meat (Chapter 3), and also represented the oxidation products of n-6 PUFA (Chapter 5). Hexanal and 1-octen-3-ol contents in meat washed by both washing solution were significantly lower ($p<0.05$) than unwashed meat since the 1st wash. The removal of these volatile compounds after washing probably due to the fact that protein can contribute to the release of volatile compounds because volatile compounds are generally bound with protein [13]. Washing, a common process in the production of fish protein gel such as surimi products, could remove not only the impurities in fish meat, but also the water-soluble protein, mainly sarcoplasmic protein [5]. Many studies have revealed the interaction between protein and flavor compounds. Perez-Juan *et al.* [14] reported that sarcoplasmic proteins were able to bound with volatile compounds at higher level than myofibrillar proteins. Therefore, washing away those protein during process could result in removing the volatile compounds binding with those proteins as well. Moreover, the washing solution added during process could lower the proteins concentration, resulting in the weakening of protein binding ability, which could also promote the release of volatile compounds [7].

However, the level of volatile compounds in rabbit fish washed meat remained constant after the 1st wash with no significant difference caused by washing times. Zhou *et al.* [7] reported the washing times affected the removal rate of aroma active compounds in silver carp water washed meat (*Hypophthalmichthys molitrix)* but not to the same extent for different compounds. Their study revealed that increase the washing times from two to three times could not reduce the concentration of some volatile compounds, such as hexanal, heptanal, 2-ethylfuran, carbon disulfide, and dimethyl trisulfide, while the levels of some another compounds, such as octanal, (E,E)-2,4-heptadienal, nonanal, 1-octen-3-ol, and trimethylamine, could be decreased after three times of washing. These results suggested that protein could selectively interact with volatile compounds [13]. On the other hand, for high lipid contents fish such as horse mackerel (*Trachurus trachurus*) in Eymard *et al.* [6] study, washing times seemed to affect the contents of VLOP by promoting lipid oxidation during washing process, which resulted in the higher levels of VLOP, such as 1-penten-3-ol and 2,4-heptadienal, after three times washing.

6.3.2 Characterization of washed rabbit fish minces during cold storage

Figure 6-7 to Figure 6-9 show the chromatogram of volatile compounds obtained from washed rabbit fish meat with different washing solution, including water and 0.5% sodium ascorbate, during cold storage. The change of volatile compounds in meat during washing process were also presented by the concentration (ng/g) of hexanal (Fig 6-4) and 1-octen-3-ol (Fig 6-5). Although, there was no significant difference between the levels of hexanal and 1-octen-3-ol in water and ascorbate washed rabbit fish meat at day 0 of storage (Fig 6-2 and Fig 6-3), the concentration of these 2 compounds in water washed meat increased significantly ($p<0.05$) when the storage time increased, while those levels in ascorbate washed meat remained constant during 6 days of storage as shown in fig 6-4 and fig 6-5.

Hexanal and 1-octen-3-ol are the secondary volatile lipid oxidation products of n-6 PUFA, which is an abundant PUFA observed in rabbit fish meat [15]. Moreover, 1-penten-3-ol, which is the VLOP of n-3 PUFA, was also found only in water washed meat during storage (Fig 6-6). The formation of these volatile compounds in water washed meat indicated the occurrence of lipid oxidation, which could be prevented by adding 0.5% sodium ascorbate as an antioxidant in washing solution. The addition of 0.2% sodium ascorbate and 0.2% sodium tripolyphosphate in washing solution also prolonged the shelf life of Atlantic mackerel fillets (*Scomber scombrus*) compared to water washing [9]. Washing horse mackerel (*Trachurus trachurus*) with various types of antioxidant solutions, such as caffeic acid and propyl gallate, also resulted in the less oxidation of the washed meat during storage [10]. The effect of other antioxidant solution on the volatile compounds prevention deserves further attention.

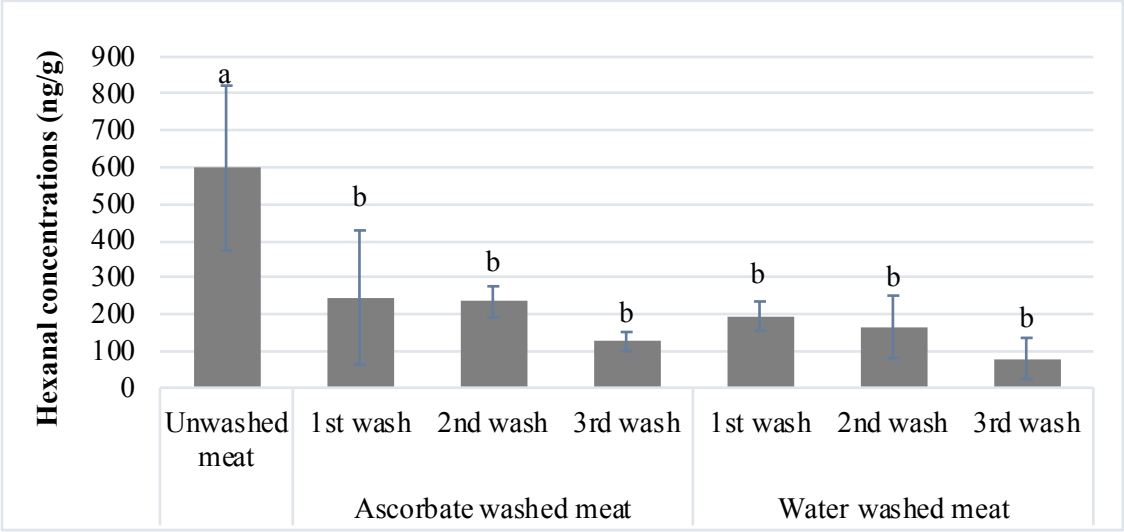

Figure 6-2 Concentration of hexanal in rabbit fish mince during washing with different washing solution

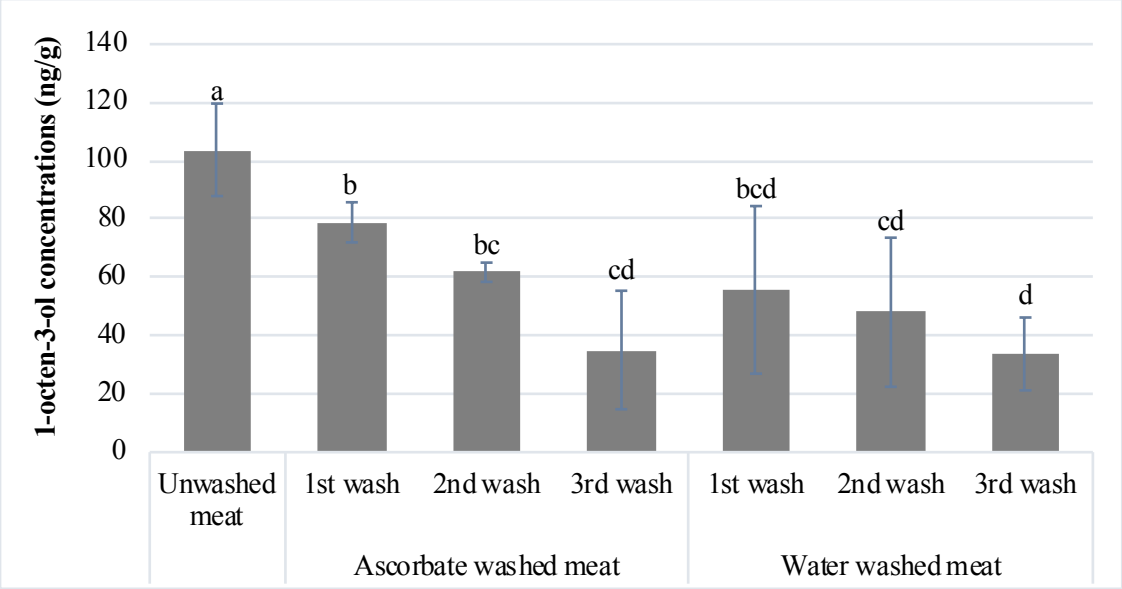

Figure 6-3 Concentration of 1-octen-3-ol in rabbit fish mince during washing with different washing solution

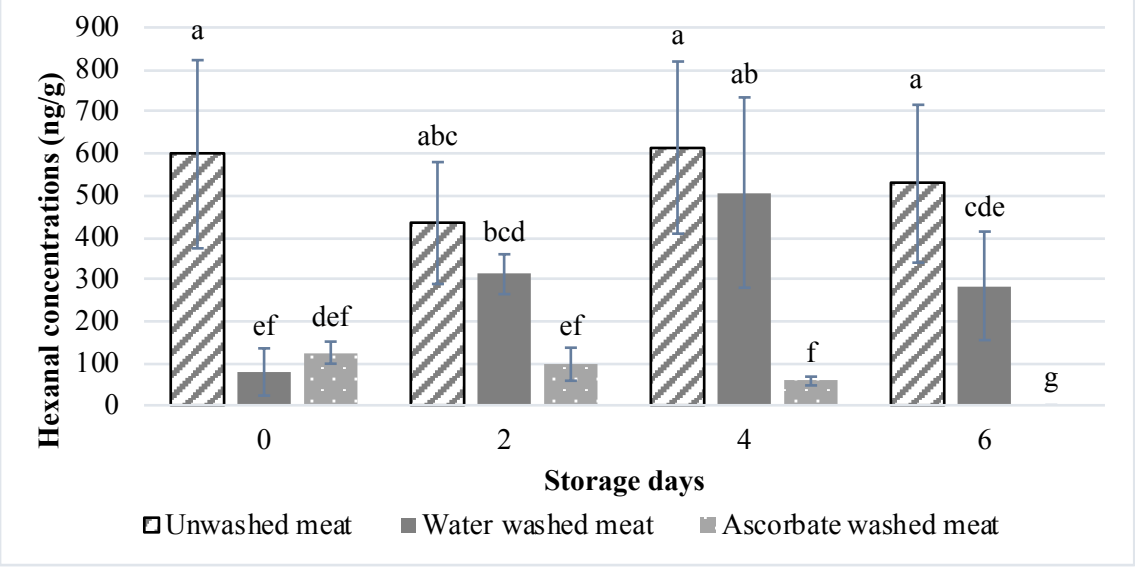

Figure 6-4 Concentration of hexanal in rabbit fish mince during storage

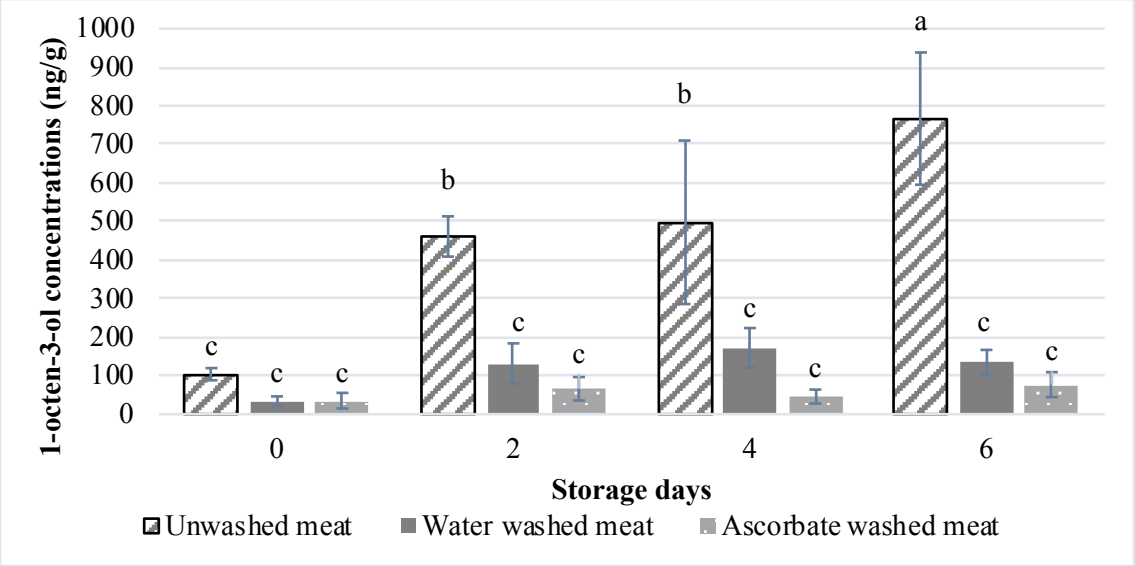

Figure 6-5 Concentration of 1-octen-3-ol in rabbit fish mince during storage

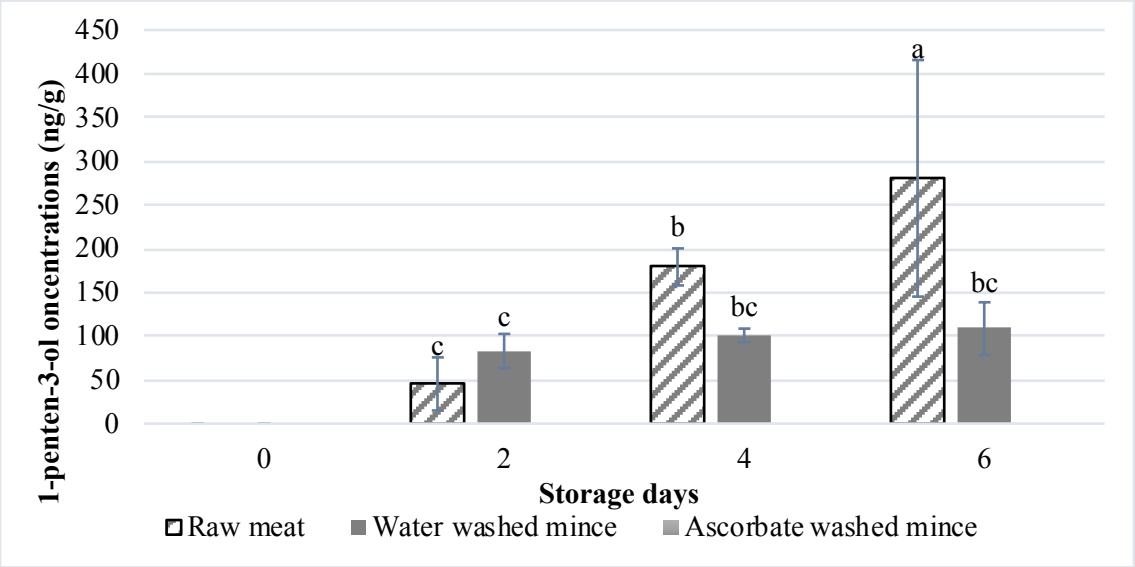

Figure 6-6 Concentration of 1-penten-3-ol in rabbit fish mince during storage

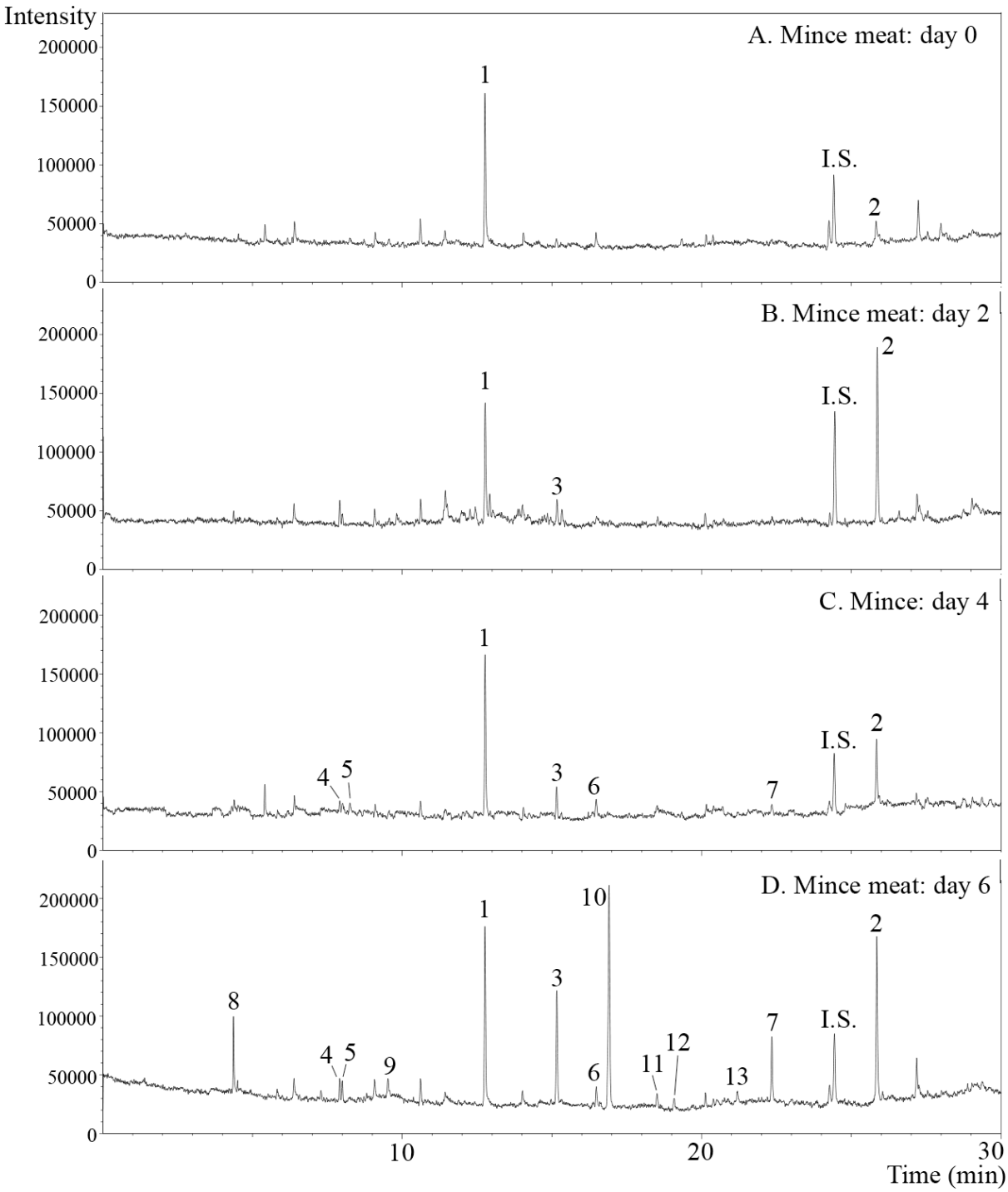

Figure 6-7 Chromatograms of volatile compounds obtained from rabbit fish meat during storage. Peak number are identified as in Table 6-2

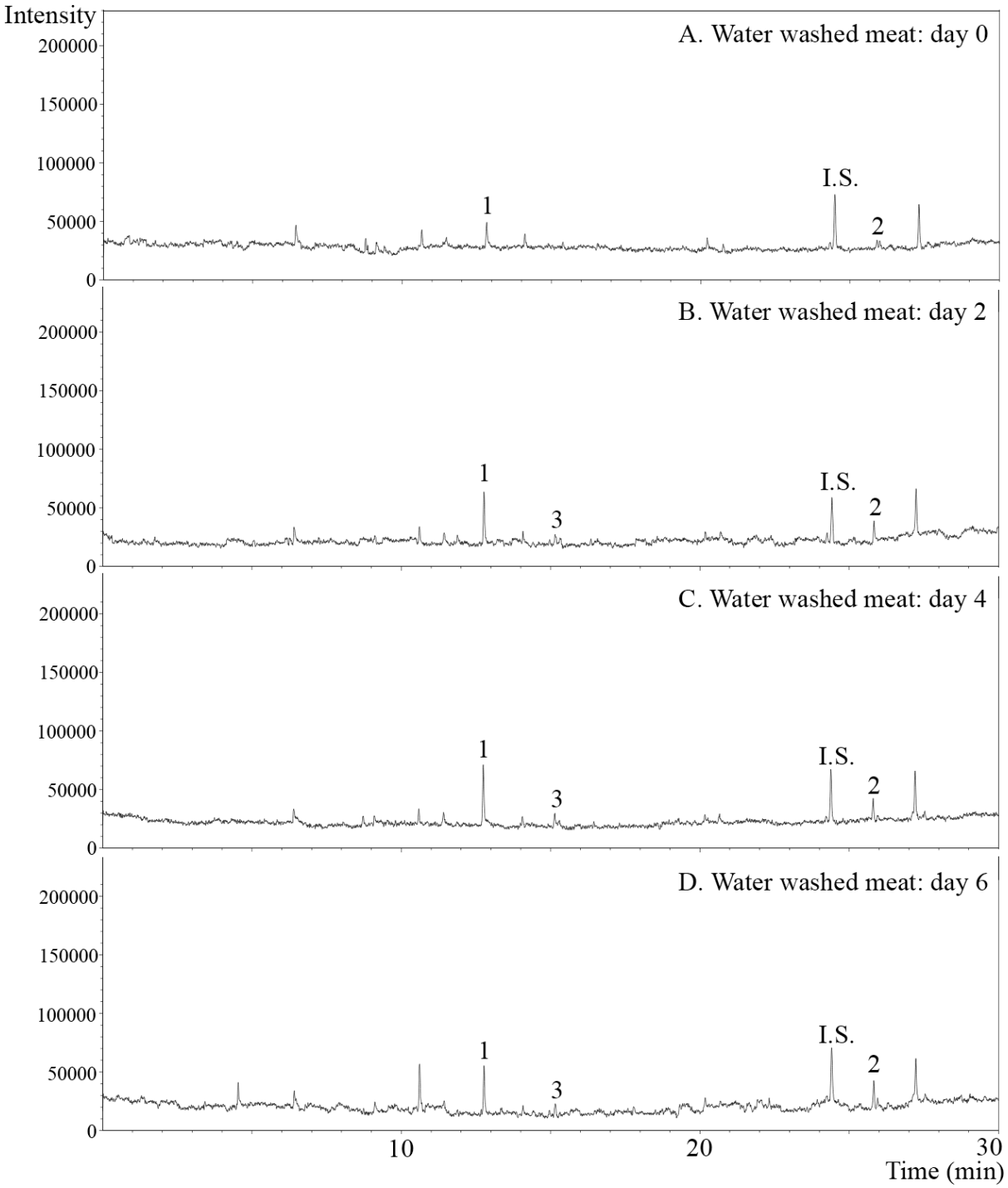

Figure 6-8 Chromatograms of volatile compounds obtained from rabbit fish water washed meat during storage. Peak number are identified as in Table 6-2

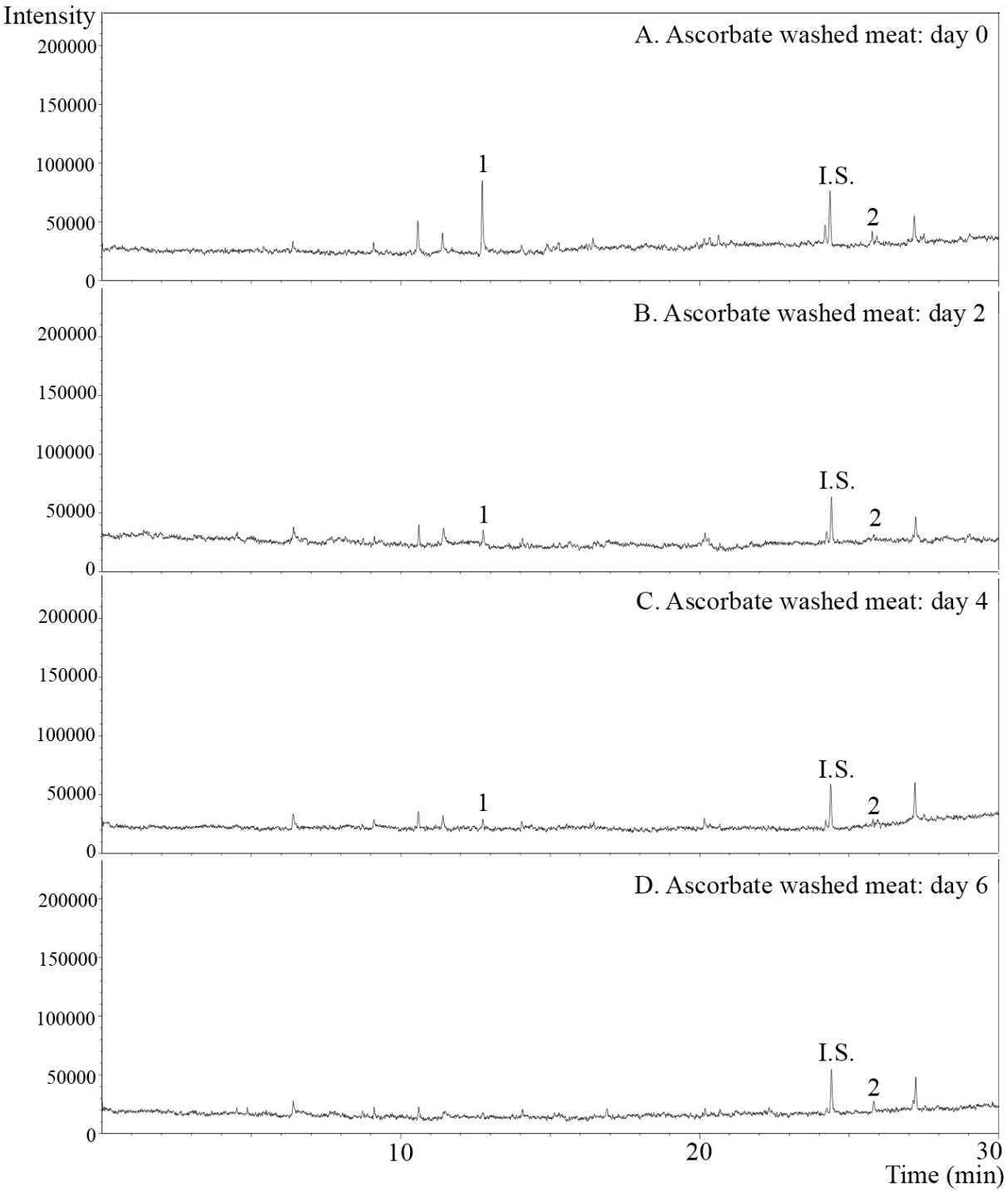

Figure 6-9 Chromatograms of volatile compounds obtained from rabbit fish ascorbate washed meat during storage. Peak number are identified as in Table 6-2

6.4 Conclusion

Washing rabbit fish mince by both water and 0.5% sodium ascorbate solution could remove the concentration of 2 key odor compounds associated with the unpleasant smell in rabbit fish, including hexanal and 1-octen-3-ol. Moreover, the generation of VLOP during cold storage could be prevented by adding 0.5% sodium ascorbate as an antioxidant in washing solution. This method is expected to be an effective method for removing the unpleasant smell in rabbit fish meat, which may lead to the increase of their potential use for manufacturing, and be a successful method for managing and restoring seaweed beds.

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General conclusion

Rabbit fish, a herbivorous marine fish with a low commercial value due to its unpleasant smell, has been considered as one of the significant causes involved in the reduction of seaweed beds, which seriously occurred in coastal areas of Japan. Therefore, this research aimed to develop the method for preventing or removing the unfavorable smell in rabbit fish meat for its wider utilization in food manufacturing, which may be a potential method to manage and restore seaweed beds.

In order to develop the method for removing or preventing the unpleasant smell, the information about the volatile compounds associated with smell and their generation mechanism is needed. The volatile compounds in rabbit fish muscle, viscera, skin and stomach contents were determined using SPME-GC-MS in chapter 3. The key volatile compounds contributing to the overall smell of rabbit fish muscle were hexanal and 1-octen-3-ol, which could be classified as volatile lipid oxidation products (VLOP) from PUFA. Various types of carbonyls, alcohols, amines and sulfur-containing compounds were found in another 3 tissues. The highest level of OAV in these 3 tissues were found at 1-octen-3-one and 1-octen-3-ol, suggesting their strong association with the overall smell of whole fish. Furthermore, after storage the fish meat at 4 °C for 6 days, the meat that was stored as whole fish deteriorated more rapidly than stored as fillets, suggesting the importance of early evisceration on the smell quality of rabbit fish.

Because the key odor compounds in rabbit fish were VLOP, lipid profiles in rabbit fish tissues were examined in chapter 4 with a special focusing on PUFA. Rabbit fish lipids contained high levels of PUFA, especially ARA, which differed from other carnivorous and omnivorous marine species that consist of mainly DHA and EPA.

The results from previous chapter led to the possibility that rabbit fish tissues may contain LOX, which involved in the formation of volatile compounds associate with the unpleasant smell in its tissues. Therefore, lipid oxidation model of crude enzyme extract and PUFA were used to investigate the generation of volatile compounds products in chapter 5. Lipid oxidation model mixtures were prepared by mixing crude enzyme extracted with commercial PUFA, including ARA, linoleic acid (LA), DHA and EPA in sodium phosphate buffer (pH 7.4). The results indicated that crude enzyme extracted from rabbit fish viscera may contain LOX because various volatile compounds associated with the fresh fish smell, such as 5-, 6-, 8-carbon compounds were generated from the reaction with PUFA. These compounds were also similar to the key odor compounds in rabbit fish tissues. Highest level of LOX activity ($p<0.05$) was clearly observed on ARA than EPA and DHA. The result suggested that the presence of the unpleasant smell in rabbit fish was probably due to LOX in its tissues, which was capable to catalyze the oxidation of PUFA, especially ARA, and led to the formation of volatile lipid oxidation products that contribute to the unpleasant smell of rabbit fish.

Lastly, in chapter 6, washing, which is an essential method used for removing the water-soluble protein and other impurities to concentrate the myofibrils in the fish protein gel production, was used to investigate the quality of rabbit fish meat during washing and storage was investigated with special focus on the removal of volatile compounds. Washing rabbit fish mince by both water and 0.5% sodium ascorbate solution could remove the concentration of 2 key odor compounds associated with the unpleasant smell in rabbit fish, including hexanal and

1-octen-3-ol. Moreover, the generation of VLOP during cold storage could be prevented by adding 0.5% sodium ascorbate as an antioxidant in washing solution. These suggested that washing with antioxidant solution is an effective method to remove the key odor compounds associated with the unpleasant smell in rabbit fish meat and also able to retard the generation of those compounds during cold storage.

Washing method developed in this study is expected to be an effective method for removing the unpleasant smell in rabbit fish meat, which may lead to the increase of their potential use for manufacturing, and be a successful method for managing and restoring seaweed beds

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