SPICES TO PREVENT LIPID OXIDATION AND IMPROVE ACCEPTABILITY IN OVEN-DRIED FISH

by

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A Thesis
Submitted to the
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of
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in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Nutrition

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at George Mason University

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

1,1,3,3-Tetramethoxypropane Equivalent....TMPE
1,1,3,3-Tetramethoxypropane.... TMP
2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid).... ABTS
Butylated Hydroxyanisole .... BHA
Dimethylamine.... DMA
Docosahexaenoic Acid....DHA
Eicosapentaenoic Acis .... EPA
Fatty acid methyl esters .... FAMES
Flame Ionization Detector.... FID
Food and Agricultural Organization ....FAO
Gallic Acid Equivalence .... GAE
Gas Chromatography .... GC
Hydrogen Peroxide Equivalent .... HPE
Malondialdehyde.... MDA
Monounsaturated fatty acid.... MUFA
Oxygen Radical Absorbance Capacity .... ORAC
Peroxide Value.... PV
Polyunsaturated fatty acid.... PUFA
Reactive Oxygen Species....ROS
Saturated fatty acid .... SFA
Thiobarbituric acid reactive substances ....TBARS
Thiobarbituric acid....TBA
Thiobarbituric acid....TBA
Total Phenolic Content .... TPC
Trichloroacetic acid ....TCA
Trimethylamine-n-oxide .... TMAO
Trolox Equivalent Antioxidant Capacity .... TEAC
Trolox Equivalent .... TE
US Department of Agriculture....USDA

## ABSTRACT

SPICES TO PREVENT LIPID OXIDATION AND IMPROVE ACCEPTABILITY IN OVEN-DRIED FISH

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This thesis describes the use of clove water extracts as antioxidants to prevent lipid oxidation in oven-dried omena fish. A 4 × 2 complete experimental design was used. Four clove-water extract doses (0 g/L, 2 g/L, 10 g/L and 20 g/L) and two oven-drying conditions (150 ℃/ 30 min and 75 ℃/ 3 h) were studied. The clove-water extract was analyzed for antioxidant capacity and the oven-dried omena fish was analyzed for lipid oxidation and fatty acid composition. Sensory evaluation was conducted using two cracker products made from the oven-dried omena fish. The results show that, comparing with directly oven-drying at 75 ℃ for 3 h, soaking in 10 g/L clove-water extract for 1 h and oven-drying at 150 ℃ for 30 min can significantly (does p-value need to be inserted in an abstract since you're talking about significance?) reduce the lipid oxidation in omena fish, and improve sensory characteristics, and may protect the omega-3 fatty acids from losses during processing. Based on these results, the pretreatment of omena with

clove extract is recommended prior to drying the fish for preparation of samples in any clinical trial designed to test health outcomes of repeated consumption of omena fish products.

# CHAPTER ONE LITERATURE REVIEW

## Introduction

Pregnant women in low income families have higher risk of nutrient deficiency due to limited nutrition and food supplies (National AIDS and STI Control Programme, 2009). As a result, their diet may not provide enough nutrients to meet pregnancy related nutrition demands, and it may increase the risk of inadequate weight gain during pregnancy, low birth-weight babies, prematurity and even fetal death and childhood death (Steketee et al., 1996; Taha et al., 1995; Temmerman et al., 1994). Animal-source food can provide complete protein and a variety of micronutrients, and can improve health outcomes for pregnancy. However, the availability of good animal-source food is limited in low income families in Kenya (Kamau-Mbuthia & Elmadfa, 2007). Thus, it becomes important to better utilize local, affordable, and edible animal resources.

Fish provides complete protein with ten essential amino acids, polyunsaturated fat, especially essential omega-3 fatty acids, and a variety of micronutrients including vitamins A, B, D and minerals such as calcium, potassium, phosphorus, iron, copper and iodine (Ackman, 1988; Gordon & Ratliff, 1992; Huss, 1995; Eddy E. Owaga, Onyango, & Njoroge, 2010). Small-pelagic fishes, which live in the pelagic zone of a water ecosystem, are the most important commercial fisheries which are provided by major water bodies in African countries (Kirema-Mukasa, 2012). Lake Victoria provides the most fisheries for Kenya, Tanzania and Uganda. The major small pelagic fish species in

Lake Victoria are *Lates niloticus* (Nile perch), *Rastrineobola argentea* (omena or dagaa), *Oreochromis niloticus* (tilapia), and several species from the genera *Haplochromis*, *Bagrus*, *Clarias*, *Synodontis* and *Protopterus* (Kirema-Mukasa, 2012).

Omena (silver cyprinid, *Rastrineobola argentea*), which is also commonly called dagaa in other African countries, is a sardine-like small fresh water fish species and an important edible small pelagic fish species of Lake Victoria, and it accounts for 60% of Lake Victoria fishery (LVFO, 2008). Omena feeds on small zooplankton and insects and is heavily preyed upon by birds and other predatory fishes. Omena is often made into sun-dried, deep-fried and hot-smoked fish products (Kirema-Mukasa, 2012). It is also made into fish powder and added as ingredients to make healthy food (Kirema-Mukasa, 2012). The fish is caught by light fishing using hurricane pressure lamps to attract fish (Kirema-Mukasa, 2012). Fresh caught fish are directly dried under the sunlight. The sun-drying process usually takes 1 day or more depending on the weather (Reynolds, 1993). The sun-dried omena is used for human food consumption in low-income families and feeding animals (Owaga et al., 2010). The quality of dried fish products is largely influenced by the undesirable flavor generated during processing. The off-odor and off-flavor is mainly caused by the biochemical reactions in fish lipid (lipid oxidation) and protein.

Spices have been used as seasoning and preservatives. Most spices contain polyphenols that have antioxidant capacity, and studies have shown that the spice antioxidants can be used to preserve meat products as a way of preventing lipid oxidation. The objective of this research is to develop the technology of using spices in preventing

fish lipid oxidation during oven drying, to ultimately prevent "fishy" odor and flavor development and improve acceptability.

## Omena Composition and Nutritional Value

Fish provides an animal source of protein and micronutrients that are important in the human diet (Kabahenda et al., 2011). Small-pelagic fishes, especially omena, have much higher availability in Lake Victoria areas than other animal protein sources.

Sun-dried omena is the most common omena product which has a long shelf-life (Kirema-Mukasa, 2012). It is rich in unsaturated fat, iron, zinc, calcium (Kabahenda et al., 2011), see **Table 1**.

**Table 1 Proximate composition of unsalted sun-dried omena from the market**

| Nutrient | Nutrients per 100 g sun-dried omena |
|---|---|
| Moisture content | 12.4% |
| Fat | 12.5-13.2% |
| Protein | 53.0-58.8% |
| Ash | 13.5-15.66% |
| Iron | 8.2-10.7 mg |
| Zinc | 10.1-10.2 mg |
| Calcium | 1556-1866 mg |

*Sources: Kabahenda et al., 2011; Bille & Shemkai, 2006; Owaga et al., 2010.

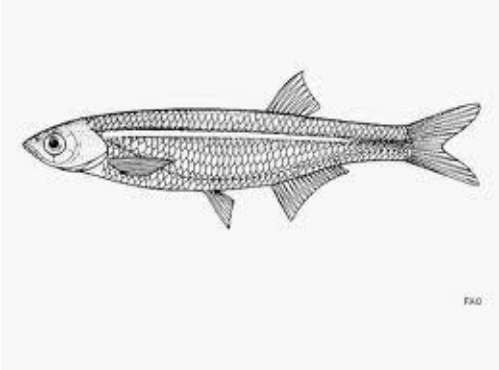


**Figure 1 Omena/ Dagaa (Rastrineobola argentea)**
Source: FAO document.

The composition of one certain fish species varies from season to season, due to the amount and quality of food that the fish eats and the amount of movement it makes (Kirema-Mukasa, 2012). During spawning period, fish usually stop feeding and live on fat and protein that were previously stored in the body. When overcrowded and the omena cannot get enough food, fish composition will change accordingly. Kenya has a closed fishing season for omena on Lake Victoria from April to the end of June annually (Kirema-Mukasa, 2012).

## Protein

Protein usually provides 15-20% of fish body weight (on a wet weight basis). In some species, the amount of protein is higher than other species. The protein content of omena is 19.1 - 21.7% on a wet weight basis (Ogonda, 2014). In **Table 1**, the crude protein content of sun-dried omena is 53-58.8% (Kabahenda et al., 2011).

Fish proteins are made up of different types of amino acids, and certain of them are essential in the human diet for maintaining a good health condition. Two essential amino acids, lysine and methionine, are found in high concentrations in fish proteins.

Comparing with meat, milk and eggs, fish protein can favorably provide an excellent combination of amino acids which is efficiently suited to human's nutritional requirements (Burt & Murray, 2001).

## Fat

Fish contains a large amount of unsaturated fats, and the type and amount of fats vary widely across species. The fat content in omena is 1.77 - 3.40% on wet weight basis (Ogonda, et al., 2014). The total fat detected in sun-dried omena is 12.5-13.2%, see **Table 1** (Kabahenda et al., 2011).

Omena is considered a fatty fish, in which the fat content is greater than 2% of body weight on wet basis (Ogonda, et al., 2014). In fatty fish, the fat content varies due to season changes and food availability (Burt & Murray, 2001). The fat content of sardines, sprats and mackerel are heavily influenced by seasons. The fat content is in negative association with water content, and in a fatty fish, the sum of water and fat percentages is fairly constant at about 80%. The fat distribution in fish flesh is not always uniform in fatty fish. For example, the fat in muscle around the head is nearly twice of that in tail muscle in Pacific salmon (Burt & Murray, 2001).

## Carbohydrates

In fish muscle and flesh, the amount of carbohydrates is usually less than 2% which is not considered significant source in human diet.

## Micronutrients in fish

Micronutrients, vitamins and minerals, are important for human in promoting good health and maintaining life. As introduced in former text, fish can provide a well-balanced supply of minerals in a bioavailable form for humans. The mineral known

composition of sun-dried omena is provided in **Table 1**. 100 g sun-dried omena contains 8.2-10.7 mg of iron, 10.1-10.2 mg of zinc and 1556-1866 mg of calcium (Kabahenda et al., 2011).

The vitamin content varies in individual fish of the same species and in different parts of the same fish. According to the FAO, the fish liver and gut contain much greater quantities of fat-soluble vitamins (Vitamin A and Vitamin D) than the flesh; water-soluble vitamins are also uniformly distributed in flesh, skin, liver and gut. The flesh usually contains more than half the total water soluble vitamins. Fish are good source of vitamin A, D and B (Kabahenda et al., 2011).

## Fishy Smell Mechanisms and Prevention Methods

Aroma compounds play an important role in the sensory characteristic and overall quality of fish products since they largely influence the consumer acceptance (Selli, Prost, & Serot, 2009). Some aroma substances are appealing; some will lead to off-flavor of products. The aroma components, including appealing odors and "fishy" odors, are mainly generated from lipid oxidation, enzymatic reaction, microbial action. The off-odor of fish products is mainly the result from three processes, automatic lipid oxidation, sulfur- nitrogen-containing precursor enzyme catalytic conversion, and trimethylamine oxide generates trimethylamine and dimethylamine (Wu, Tao, & Gu, 2013).

### Automatic lipid oxidation

Automatic lipid oxidation happens automatically during heating and storage. It is an oxidative degradation of polyunsaturated fatty acids caused by reactive oxygen species (ROS). The mechanism of generation of aroma components occurs when polyunsaturated,

especially omega-3 polyunsaturated, fatty acids generate carbonyl compounds during the process of autoxidation, such as 2,4 - decadienal and 2,4,7 – triene decyl aldehyde. In fresh fish, fishy flavors are reported to be caused by increasing concentration of these carbonyls (Liu, 1997).The auto-oxidation process follows the free radical chain reaction. The reaction has three periods, initiation, propagation and termination. See **Figure 2**.

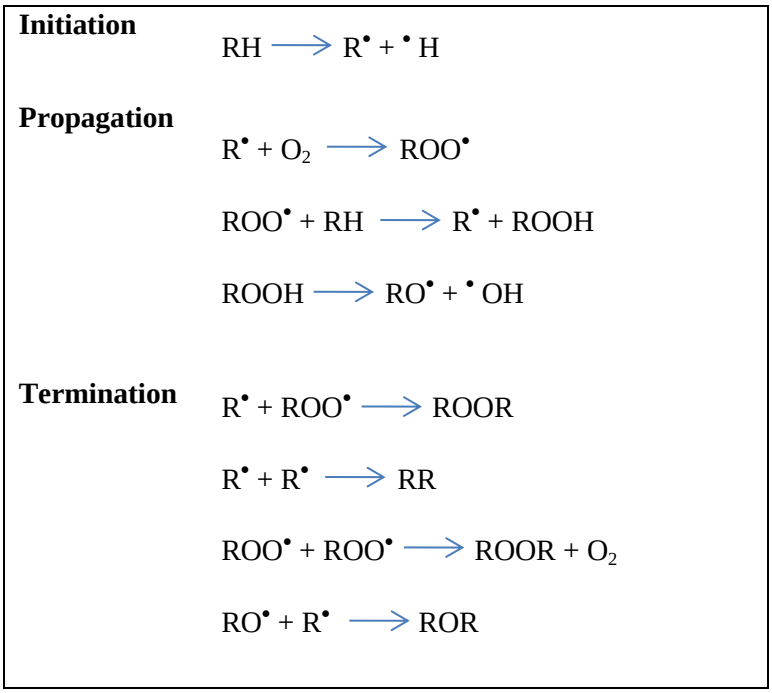



**Figure 2 Free radical chain reactions**

In the initiation period, the reactive oxygen species (ROS) react with fatty acids as initiators to seize hydrogen atom and highly unstable fatty acid radicals are generated. Fatty acid radicals attract hydrogen from another chemical species, or reacts with triplet oxygen to generate primary lipid oxidation product peroxides, including fatty acid peroxyl radical. The peroxides can be measured by peroxide value (PV) assay. In the

termination period, the fatty acid peroxyl alkyl radical ROO• reacts with lipid radical R• to form relatively stable dimers ROOR or peroxides break down to form relatively unreactive non-radical secondary products, such as aldehydes, ketones, organic acids, and hydrocarbons. The secondary product malondialdehyde (MDA) and others can be measured by the thiobarbituric acid reactive substances (TBARS) assay. The process is seen in **Figure 3**.

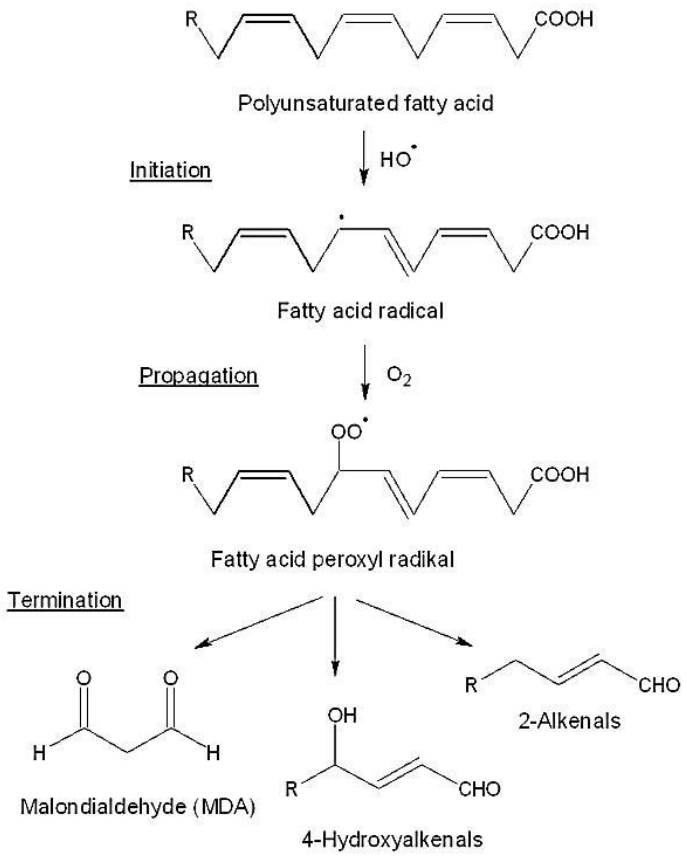



**Figure 3 Fatty acids free radical chain reaction**
Source: Mimica-Duki et al., 2012.

### Sulfur-nitrogen-containing precursor enzyme catalytic conversion

Volatile sulfur compounds, which are other causes of undesired fishy odor, result from microbial degradation of free cysteine and methionine ($C_5H_{11}NO_2S$) in fish muscle. The catalytic conversion effects on characteristic flavor precursor compounds are mainly found in fish skin mucus and blood, such as δ- amino acid, δ- amino-pentanal and Hexahydropyridine (Wu et al., 2013).

### Trimethylamine oxide generates trimethylamine and dimethylamine

Trimethylamine-n-oxide (TMAO) is present in most marine fish (salty water fish) (Oetjen & Karl, 1999). It can be reduced to trimethylamine (TMA) by spoilage bacteria, which raises the characteristic fishy odor of iced fish. Therefore, TMA is used as an indicator for spoilage. Dimethylamine (DMA) is reduced from TMAO by an intrinsic enzyme activity in fish during frozen storage of some fish species, when bacterial growth is inhibited (Pedrosa-Menabrito & Regenstein, 1990). DMA is only generated during frozen storage with sub-zero temperature (Sotelo, Gallardo, Piñeiro, & Pérez-Martin, 1995); thus, DMA is used as an indicator for frozen storage. TMA and DMA, degraded from TMAO, have been used as quality indicators for decades (Timm & Jørgensen, 2002).

### Earthy and muddy flavor caused by water environment

There is also an important flavor in aldehydes active substance E-2- nonenal, which is described as fishy and earthy. It is caused by the imbalance between algae, molds and bacteria. This kind of fishy flavor is mostly found during summer due to excessive amounts of nitrogen and phosphorous in the water system (Kaewplang, 2005).

## Spice composition and applications in food and medicine

Spices are commonly used as a food adjunct and added to dishes to provide flavor or used as preservatives to prolong the shelf-life all around the world, especially in India, China, and many other southeastern Asian countries, for thousands of years (Srinivasan, 2005). The most commonly used spices include cinnamon, rosemary, cloves, ginger, cumin, and oregano. Spice plants belong to botanical families, which include *Labiatae/ Lamiaceae* (e.g., rosemary, oregano, and sage), *Lauraceae* (e.g., cinnamon), *Peperaceae* (e.g., black pepper), *Myrtaceae* (e.g., clove), and *Umbelliferae* (e.g., cumin). Most spice plants normally grow in tropical and temperate areas (Kumar, 1997; Srinivasan, 2005).

Interestingly, spices are also important traditional medicines throughout human history. Many spices have exhibited the medicinal properties of anti-inflammatory, antimicrobial, anti-carcinogenic, hypolipidemic, antimutagenic, and anti-oxidative effects (Aruoma et al., 1996; Dorman & Deans, 2000; Lampe, 2003; Srinivasan, 2005).

## Spices as Antioxidants

Spice crude extracts (50% alcohol extract) are reported to contain a variety of antioxidants, and could significantly inhibit enzymatic lipid peroxidation (Shobana & Akhilender Naidu, 2000). Using natural antioxidants is effective and has been described as one of the most technically simple ways to prevent lipid oxidation (Tomaino et al., 2005). Certain spices and spice extracts contain components with anti-oxidative capacity. Compared with cinnamon, nutmeg, basil, oregano and thyme, when maintained at room temperature or even heated at 180 ℃ for 10 min, clove had the highest DPPH radical scavenger activity (Tomaino et al., 2005). A study was conducted by Shan et al.(2005) to compare the antioxidant capacity of 26 commonly used spices (clove, coriander, parsley,

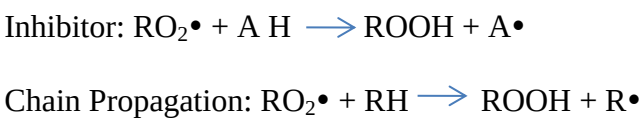
cumin, rosemary, thyme, sage, oregano, nutmeg, white pepper, black pepper, ginger, bay, green cardamom, caraway, cinnamon, cinnamon stick, poppy, green peppercom, chinese prickly ash, chilli, dill, lemon grass, star anise, mint and sweet basil). The phenolic content of 26 spices ranged from 0.04 to 14.38 g Gallic Acid Equivalence (GAE)/ 100 g of dry weight (DW), the mean total phenolic content (TPC) value of all 26 tested spices was 3.26 g GAE/ 100 g of DW. The trolox equivalent antioxidant capacity (TEAC) of 26 spices ranged from 0.55 to 168.7 mmol/100 g of DW, and the mean TEAC value of 26 tested spices was 31.7 mmol/100 g of DW. Among 26 tested spices, cloves showed the highest TPC value of 14.38 g $\pm$0.006 of GAE/ 100 g of DW and the highest TEAC value of 168.66 $\pm$0.024 mmol of trolox / 100 g of DW. The major phenolic compounds of clove are phenolic acids (gallic acid), flavonol glucosides, phenolic volatile oils (eugenol, acetyl eugenol), and tannins (Shan et al., 2005).

Spices have good antioxidant properties and can be efficiently used to control lipid oxidation in the food industry (Madsen & Bertelsen, 1995). Oxidative reactions are defined by their transfer of electrons between atoms or molecules. Oxidation will result in development of off flavors and odors, discoloration, texture, and nutritional value losses. Oxidation reactions are usually classified by causes, including oxygen, light, heat, heavy metals, etc. Alkaline conditions and degree of unsaturation will also influence an oxidation reaction (Frosch, 1998). Polyunsaturated fat is more likely to be oxidized than monounsaturated fat due to the lower required energy to break a double bond. It is important to choose the proper antioxidants based on different mechanisms of action to achieve optimal antioxidant effects in a food product (Frosch, 1998). There are three

categories of antioxidants reaction, according to their mechanism: (1) free radical terminators, (2) oxygen scavengers, and (3) chelating agents.

**Free Radical Terminators**

Free radical terminators work by donating a hydrogen ion from phenolic hydroxyl group to stabilize free radicals and interrupt the free radical chain reactions (Sherwin, 1978). This reaction was firstly demonstrated by Bolland and Ten-Have (1947).




Antioxidants react with peroxy radicals ($RO_2\bullet$) but not lipid alkyl radicals ($R\bullet$). Antioxidants (AH) act as the inhibitor or free radical acceptors to terminate the free radical chain reaction by donating hydrogen. The free radical termination is postulated to be a competition between the inhibitor reaction and the chain propagation reaction (Boland & Ten-Have, 1947).

By this mechanism, antioxidants prevent the formation of hydroperoxides and accelerate the termination of the free radical chain reaction. It is important to mention that, in this mechanism, antioxidants only inhibit the propagation step from continuing (Naidu, 1995; Reddy & Lokesh, 1992), but free radical terminators do not prevent the initial free radical generating step. They scavenge the radicals to mitigate the damage (Tyler, 1975; Yang, Yasaei, & Page, 1993). Commonly used free radical terminator

antioxidants in foods include butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), propyl gallate (PG) and tocopherols (Frosch, 1998).

#### Oxygen Scavengers (Reducing Agents)

Oxygen is an essential reactant in lipid oxidation as shown in the propagation stage in **Figure 3**. Oxygen scavengers are reducing agents that competitively react with oxygen by transferring hydrogen atoms. Commonly used oxygen scavengers in food are ascorbic acid, ascorbyl palmitate, sulfites, glucose oxidase, and erythorbic acid (Frosch, 1998).

#### Chelating Agents

Chelators contain unbounded electrons in their molecular structure, allowing them to form stable complexes with reactive metals, such as iron and copper. The formation of these complexes can reduce the opportunity of the metals to initiate oxidation reactions. Citric acid, ethylenediaminetetraacetic acid (EDTA), and various polyphosphates are commonly used chelating agents in foods (Frosch, 1998).

### Antioxidant stability during heating

Spices are commonly consumed as fresh ingredients by adding shortly before or after cooking up to 100 ℃. One study showed that the antioxidant activity of selected spices, including cloves, garlic, ginger, cinnamon, and pepper, significantly increased after keeping them in boiling water for 30 min (Shobana & Akhilender Naidu, 2000). However, the antioxidant thermal stability varies across different spices under different

cooking conditions. Turmeric, capsicum, and pepper (mixed spices) were reported to have significant active principle component loss when boiled with the souring agent tamarind (Srinivasan et al., 1992). According to thermal stability studies on spices, garlic and crude gingerol can even resist heat of 105-165 ℃ and maintain significant antioxidant activity when cooked in soybean and ground nut oils (Gazzani et al., 1998). To our knowledge, there is no data on cloves heated at temperatures above 100 ℃. However, according to Shobana and Naidu (2000), clove antioxidant components are stable in boiling water for at least 30 min (100 ℃).

## Methods for lipid oxidation analysis

Lipid oxidation can be either measured by assessing primary lipid oxidation products (peroxides) or assessing secondary products (carbonyls). The formation of peroxides happens with the decrease of unsaturated fatty acids or oxygen. It can be measured by the peroxide value (PV) assay. Primary products will decompose to form stable secondary products carbonyls, including malonaldehyde (MDA), hydrocarbons and fluorescent products (J. Ian Gray & Monahan, 1992). They can be detected by the thiobarbituric acid reactive substances (TBARS) assay. There are many methods for lipid oxidation measurement, but each method cannot provide a whole picture of the lipid oxidation process. Gas chromatography (GC) is widely used for determining fatty acid composition of food oils and is appropriate to assess the differences in fatty acid composition before and after the oven-drying process.

## Peroxide Value

The peroxide value (PV) test is designed to detect lipid oxidation progress by measuring concentration of primary product peroxides, which are generated during the free radical chain reaction propagation stage (see **Figure 3**). The peroxide value is widely used for testing lipid oxidative rancidity and the extent of lipid oxidation. The iodometric titration assay is the current standard method and has been commonly used in measuring peroxides in food products (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). This method is a discoloration reaction using starch as an endpoint indicator. It is based on oxidizing $I^-$ to $I_2$ with hydroperoxides (ROOH) presented in the sample and the $I_2$ is measured by titrating with sodium thiosulfate.

$$2\ I^- + H_2O + ROOH \longrightarrow ROH + 2\ OH^- + I_2$$

$$I_2 + 2\ NaS_2O_3 \longrightarrow Na_2S_2O_6 + 2\ NaI$$

However, the titration method has limitations including having difficulty with determining the endpoint, time-consuming, and labor-intensive, thus it is not appropriate when having a large sample size (Dobarganes & Velasco, 2002; Eymard & Genot, 2003; Ru ́z, Ca ̃nada, & Lendl, 2001). The ferrous oxidation of xylenol orange (FOX) assay is proposed to replace the iodometric titration method. The FOX assay is based on oxidizing ferrous ion ($Fe^{2+}$) to ferric ion ($Fe^{3+}$), and ferric ion forms a blue-purple complex with xylenol orange, which has maximum absorption at 550-600 nm (Dobarganes & Velasco, 2002; Eymard & Genot, 2003). Hydrogen peroxide can be used as a standard. As

reported by DeLong et al. (2002), the FOX assay result is in good agreement with the iodometric method; it is more rapid, sensitive and less expensive than the standard titration method and has been applied to dairy, meat, plant tissue and oil samples (DeLong et al., 2002; Dobarganes & Velasco, 2002; Eymard & Genot, 2003). Thus, the FOX method is preferred.

High peroxide values are a definite indication of a rancid fat; however, care must be taken in interpreting low PV results, because lower amount of peroxides may result from peroxide depletion to form secondary lipid oxidation products (termination stage in free radical chain reaction). Therefore, it is common to measure both PV and TBARS in the same food product.

**TBARS**

Kohn and Liversedge (1944) found that animal tissue can react with 2-Thiobarbituric acid (TBA) to form a pink complex. It was later used in measuring lipid oxidation. The mechanism involves two molecules of 2-Thiobarbituric acid (TBA) reacting with one molecule of secondary lipid oxidation product MDA to form the MDA-TBA complex (see **Figure 4**), which has a pink color. The absorbance can be measured at 532 nm to quantify MDA, according to a standard curve with known amounts.

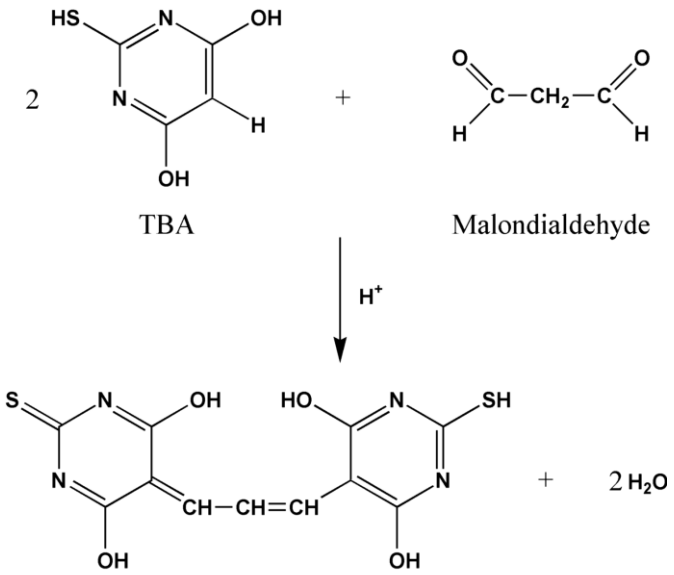



**Figure 4 Reaction between 2-Thiobarbituric acid and malondialdehyde**
Source: Rojanasasithara, 2008.

Since the original discovery, different TBARS assays have been adopted to measure the secondary products (MDA) of lipid oxidation with greater ease. The modified extraction method was first developed by Salih et al. (1987) using poultry. The lipid is extracted from animal tissue in water with a high-speed homogenizer. Comparing with other TBARS assays, the modified extraction method is widely used in recent analysis of animal tissues as an indicator of the extent of lipid oxidation because it is considered accurate, rapid, and easy to conduct (Botsoglou et al., 1994).

## Total Lipid

The Folch et al. (1957) method has been determined to be the most effective for the extraction of a broad range of lipid classes in lipoproteins (Reis et al., 2013). It was primarily used for preparation and purification of brain lipids. It has since been widely used in extracting and measuring crude lipid of animal tissue.

Lipids are present in animal tissues in a variety of physical forms. The complex lipids are usually constituents of membranes and in close association with proteins and polysaccharides, with which they interact by hydrophobic and van der Waals forces, and perhaps by ionic bonds. Various solvents or solvent combinations have been suggested for lipid extraction, but chloroform-methanol (2:1 by volume), as suggested by Folch et al. (1957), is the most commonly used. However, in the Folch method, the harmful solvent chloroform is required. For large quantity lipid extraction experiments, chloroform may cause health problems to the researchers and pollute the environment. Therefore, the modified Folch method was developed by Lin et al. (2004) to replace the original method. An Ethyl Acetate/Ethyl Alcohol mixture were used as alternative to old Folch reagents in this new method. Comparative studies on lipid extraction methods indicated that the modified Folch method was highly efficient, and as reliable as the Folch method in terms of extraction yields, lipid profiles, and fatty acid compositions (Lin et al., 2004; Ramalhosa et al., 2012).

## Gas Chromatography for Fatty Acid Characterization

Gas chromatography (GC) is widely used in identifying the fatty acid composition of oil samples. This technology uses Helium as carrier gas and a fused silica capillary column as the stationary phase. The most commonly used detector is the flame ionization detector (FID) which is most suitable for detecting carbon-carbon or carbon-hydrogen bonds (Reineccius, 2003). The FID uses hydrogen as fuel and detects compounds as they are eluted by Helium from the column.

Before a fat sample can be analyzed by GC, it needs to be saponified and esterified to form fatty acid methyl esters (FAMES) (Reineccius, 2003), see **Figure 5**. Thus, the compounds can be volatized at high temperatures of approximately 250 ℃.

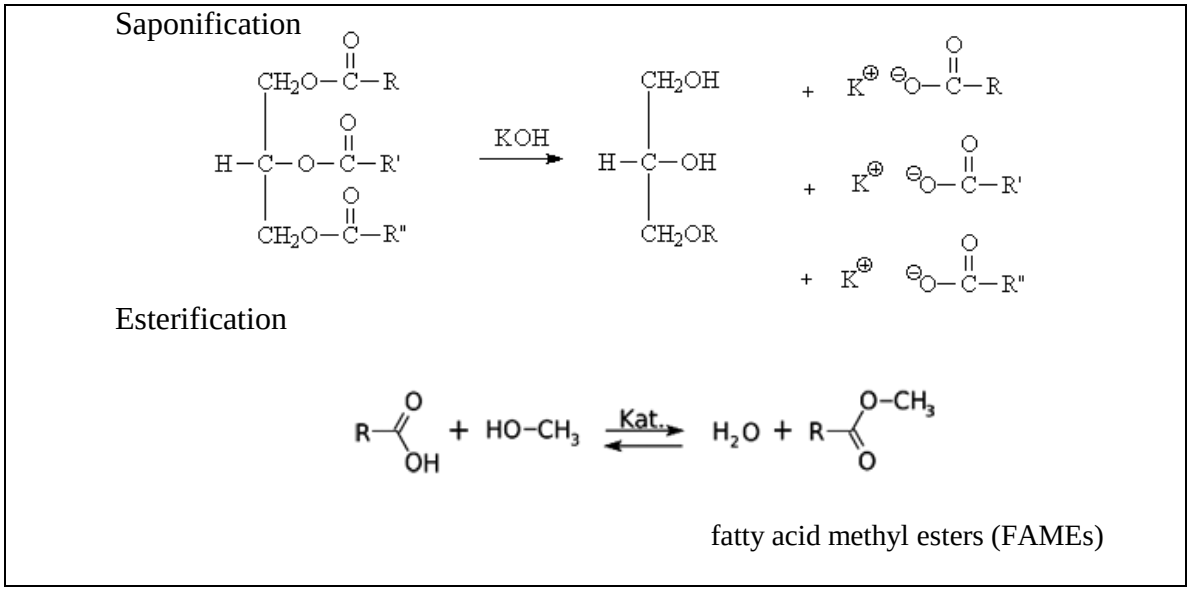



**Figure 5 Triglycerides saponification and esterification**
Source: Azaquar, 2011.

GC technology is highly sensitive in compounds analysis; however, the sample preparation is time-consuming and sample components may alter during this process (Reineccius, 2003). Thus, oxidation protection strategies, such as adding antioxidants, may be needed during preparing FAMEs samples, and synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used. The result of this analysis provides information about the composition of fatty acids in a sample.

## Methods for Antioxidant Analysis

The antioxidant capacity of a substance is the ability to prevent or delay deterioration by oxygen (Huang, Ou, & Prior, 2005). Antioxidant capacity assays are majorly based on electron transfer reaction (ET) or hydrogen atom transfer reaction (HAT). In HAT-based assays, antioxidants and substrate compete to react with thermally-generated peroxyl radicals; the oxygen radical absorbance capacity (ORAC) assay is one of them. ET-based assays measures the capacity of an antioxidant to reduce an oxidant, which includes the total phenolic content assay using Folin-Ciocalteu reagent, DPPH and ABTS radical scavenging capacity assays, SOD assay and the FRAP assay (Huang et al., 2005). There are multiple types of free radicals and different sources of antioxidants within a biological system. While several assays can be used to determine aspects of antioxidant capacity, there is not one single assay to determine total antioxidant capability (Prior, Wu, & Schaich, 2005). There is also a lack of standardization in antioxidant assays between laboratories. The results of one assay reported by a laboratory are only true under specific conditions (Huang et al., 2005). Therefore, more than two antioxidant assays are required to determine the scope of antioxidant capacity of a single compound, in an attempt to predict and understand its behavior in a complex chemical system like food.

### Total Phenolic Content

The total phenolic content assay is designed by using Folin-Ciocalteu (FC) reagent color reaction to measure the reducing capacity of antioxidant sample. The TPC assay is based on electron transfer reaction, in which phenol transforms into phenolate anion and reduces the FC reagent (Huang et al., 2005). The reaction has a color change

from yellow to blue, and the absorbance is measured at 765 nm using a spectrophotometer. Gallic acid is used as a standard and the phenolic content of clove extracts is represented by gallic acid equivalence (GAE).

This assay is widely used in analyzing antioxidants because it is fast, inexpensive, and has good correlation with other antioxidant assays. However, the reaction can be interfered by many other organic and inorganic compounds (Prior et al., 2005).

### Oxygen Radical Absorbance Capacity (ORAC)

Oxygen Radical Absorbance Capacity (ORAC) assay is designed to estimate peroxy radical scavenging activity of biological samples in vitro. It was first developed by Cao and Cutler (1993). This assay measures the peroxyl radical scavenging ability of a free radical chain-breaking antioxidant. The mechanism involves mixing the fluorescent molecule (either beta-phycoerythrin or fluorescein) with free radical generators 2,2' – azobis (2-amidinopropane) dihydrochloride (AAPH) (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002); the oxidative degradation of fluorescent molecule by AAPH at 37 ℃ is measured (Cao et al., 1993). The result is determined by calculating the area under the curve of fluorescein degradation compared with blank (Ou et al., 2002). Trolox is used as the standard.

### ABTS•+ radical scavenging assay

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid, ABTS) is commonly used to observe the reaction kinetics of specific enzymes and detect for the binding between molecules. ABTS is converted to its radical cation, $ABTS^{\bullet+}$, by addition of manganese dioxide (Miller, Sampson, Candeias, Bramley, & Rice-Evans, 1996).

In the food industry, the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation ($ABTS^{\bullet+}$) decolourisation test is widely used for assessing the antioxidant activity of various substances in foods. $ABTS^{\bullet+}$ is blue and has the largest absorbance at 734 nm wave length. $ABTS^{\bullet+}$ can react with most chemical antioxidants including phenolics, thiols, and Vitamin C. During the reaction, the blue $ABTS^{\bullet+}$ is converted back to its colorless neutral form. Thus, the loss of absorbance is measured at 734 nm using a spectrophotometer.

## Sensory Evaluation of Fish Cracker

Sensory evaluation is an important process in making new food products. It is the evoking, measurement, analysis, and interpretation of human responses to products by using senses of sight, smell, touch, taste, and hearing (Stone & Sidel, 2004). Chemical assays are commonly used for evaluating the level of lipid oxidation in meat (Laguerre, Lecomte, & Villeneuve, 2007). To be mentioned, sensory evaluation is commonly used in product evaluation since human detection of aroma compounds is much more sensitive than chemical analysis and instrument measurements. Sensory analysis is the gold standard of lipid oxidation measurements because it is the direct measurement of off-odor and off-flavor generated during oxidation (Damodaran, Parkin, & Fennema, 2008).

Sensory evaluation can be used for assessing sensory characteristics like palatability, color, flavor, mouth texture of food products (Nute, 2002). The triangle test (ISO 4120: 2004), paired preference test (ASTM E2263, 2012), and quantitative response scale test (ISO 4121: 2003-E) are commonly used to assess the perception of differences between two different samples.

In the triangle test, two potentially same samples and one different sample are provided for each participant, and the participant is asked to identify the single different sample. This assay is based on a binomial test with null hypothesis ($H_0$) stated "there are no differences between samples" and alternative hypothesis (Ha) stated "there are significant differences between samples". The triangle test requires at least 24 subjects and $H_0$ is rejected when more than 12 subjects give correct answer ($p < 0.05$) (ISO 4120:2004). A larger sample size will result in a larger power. The quantitative response scale test (ISO 4121: 2003-E) is to rate the sensory characteristics (color, flavor, odor, and texture) of one or more products on a given scale ranged from 0 to 10, where 0 corresponded to "least, not at all" and 10 corresponded to "most, extremely". The paired preference test (ASTM E2263, 2012) is used to determine an overall preference between two different samples and the subjects are forced to indicate which one they prefer.

## Preliminary Data

The acceptability of omena fish cracker among Kenyan women was previously studied (Gewa, Frankenfeld, Slavin, & Omondi, 2014). Omena fish was blanched in boiling water and oven-dried at 75 ℃, then ground into powder to mix into cracker flour. The fish-enhanced cracker had high protein content of 19.1 g per 100 g cracker and significant amount of calcium, iron and zinc. The fish cracker was liked for its crunchy texture, dark-brown color and spicy flavor; however, it was reported to have fish odor, which made it less preferred than regular wheat cracker (Gewa et al., 2014).

Several studies have indicated that clove has the highest total equivalent antioxidant capacity among different spices (Katalinic, Milos, Kulisic, & Jukic, 2006;

Shan et al., 2005). Importantly, it is also available and consumed in Kenya. Alcohol extraction, boiling water, and distillation methods are widely used in spice antioxidant extraction. However, alcohol extraction and distillation methods are more expensive than water extraction and/or require the removal of solvent prior to use in food products for human consumption, which make them incompatible for use in small production kitchens in Kenya. Furthermore, a previous study showed that boiling the spices in water for 10 minutes can extract most of the water-soluble antioxidants; the antioxidants are stable during boiling for 30 min or longer (Arabshahi-D, Vishalakshi Devi, & Urooj, 2007). Taken together, this suggests that boiling water extractions of clove are a suitable material for proof-of-concept testing.

In our preliminary work, the total phenolic content of 5 mg/mL, 10 mg/mL, 20 mg/ml, 30 mg/ mL, 40 mg/ mL and 50 mg/mL clove-water extracts were assessed. The extracts were prepared by adding 20 mg, 100 mg, 200 mg, 300 mg, 400 mg and 500 mg of ground clove (Tropical Heat, Nairobi, Kenya) into 10 mL boiling water and keeping boiling for 10 min. The total phenolic content, represented by gallic acid equivalent (GAE), increased with the amount of clove added, as shown in **Figure 6**. While overall phenolic content increased with increasing cloves extracted per unit of water, extraction efficiency decreased, as seen in **Figure 7**, with 10 mg/mL producing the most efficient extraction. Furthermore, cost-effectiveness of production of the final product must be considered at this early stage. The 10 mg/mL dose is equivalent to an extraction of 10 g of ground cloves per liter of water, which is considerably more feasible than 50 g of ground cloves per liter of water.

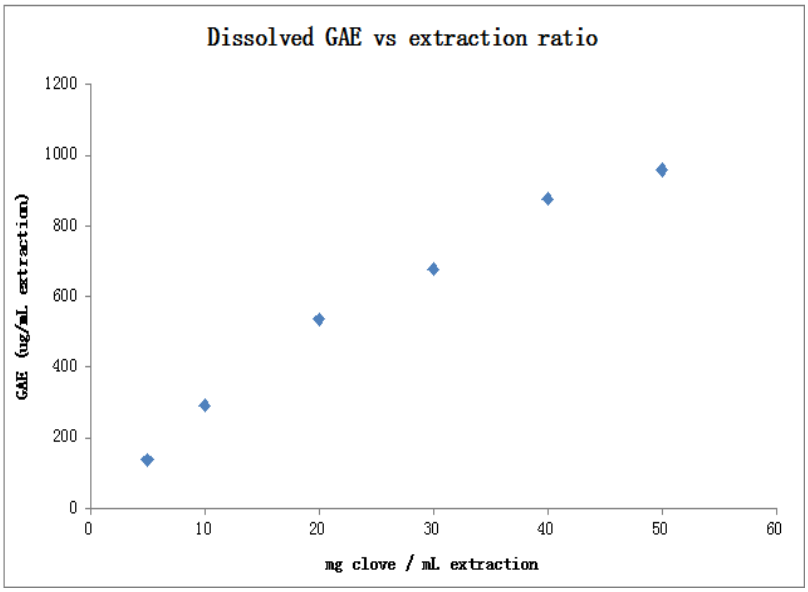



**Figure 6 Dissolved gallic acid equivalent vs extraction ratio**

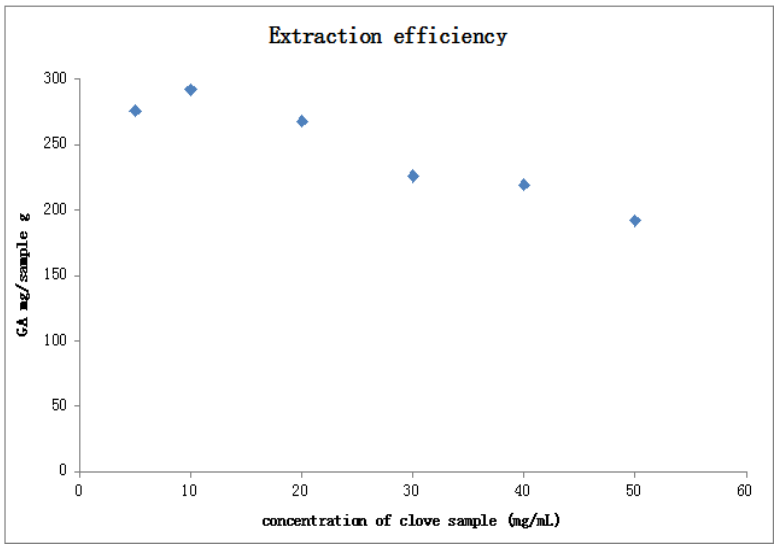



**Figure 7 Extraction efficiency of cloves at varying concentrations**

## Research Questions and Objectives

### General research question:

Can pre-treatment with spice extracts, especially clove, prevent fish lipid oxidation and off-flavor development during the oven drying process?

### Objectives:

1. To compare two oven-drying conditions (high temperature/short time vs. low temperature/long time) for impact on lipid oxidation in omena fish.
2. To evaluate the antioxidant capacity of clove water extracts and their efficiency in inhibiting lipid oxidation in oven-dried omena fish.
3. To conduct a sensory test, assessing the impact of spice extract pre-treatment on sensory attributes of a fish product related to lipid oxidation.

Fish lipid is easily oxidized when exposed to the air and heat, and the oxidation process generates an unpleasant fishy odor and negatively influences the quality of fish products. The extent of lipid oxidation is mainly determined by exposure time and temperature. A low temperature (75 ℃) and long drying period (3 day, 1 hour per day) condition is commonly used in Kenya, which results in unacceptable fishy odor and flavor. Previous studies have shown that spices contain considerable amount of phenolic antioxidants and can effectively prevent lipid oxidation in meat products. Thus, this study attempted to minimize lipid oxidation during oven-drying of fish by 1) using a different drying procedure (higher temperature 150 ℃ / shorter drying period 30 min) and 2) pre-soaking fish in clove water extract.

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# CHAPTER TWO OMENA LIPID OXIDATION AND CLOVE WATER EXTRACT ANTIOXIDANT CAPACITY

## Introduction

Omena (*Rastrineobola argentea*) is one of the most commercially important fish species, and is also an affordable alternative to other expensive animal foodstuffs in the Kenyan markets. It provides 60% of Lake Victoria fishery products (Owaga et al., 2010). As a fatty sardine-like freshwater fish, omena is considered to be a good source of high quality protein, unsaturated fatty acids, vitamins A, the B family, and D and minerals including calcium, iron, zinc, copper, iodine and potassium (Kabahenda et al., 2011; Owaga et al., 2010). Sun dried omena fish is a popular product in Kenyan markets, and has high crude protein content (60 to 75%). Omena fish meal is used in food products to enhance the nutritional value (Owaga et al., 2010). A previous study developed on omena fish cracker with 19.1% protein content, almost three times of that of the equivalent wheat cracker. However, the fish product had a lower acceptability than the wheat cracker in the target population of pregnant mothers, mainly due to the fish odor (Gewa et al., 2014).

The polyunsaturated property of fish lipid is desirable from a nutritional perspective, but makes it easier to be oxidized and turn rancid. Owaga et al. (2010) reported a statistically significant difference ($p < 0.05$) in the total fat content of fresh omena fish (14.8 % dry weight base (dwb)) vs. sun-dried omena fish (13.9% dwb). The decline of fat content during the sun-drying process and storage may result from rapid

lipid oxidation happening over time (Huss, 1995; Owaga et al., 2010). Another study showed that sun-dried omena fish had 12.5 to 13.2% fat content (Kabahenda et al., 2011). The fat content of sun-dried omena fish varies due to many environmental factors, including the change of seasons and the food supplies in Lake Victoria (Kirema-Mukasa, 2012). In other fish lipid oxidation studies, salted-wash pre-treatment before drying, in which sodium chloride played a role of pro-oxidant, led to lower crude fat content due to accelerated lipid oxidation (Marc, Kaakeh, & Mbofung, 1998; Rangoda & De Silva, 1979). The oil extracted from fresh omena fish contains 37.0% saturated fatty acids (SFA), 22.1% monounsaturated fatty acids (MUFA), 21.2% Omega-3 polyunsaturated fatty acids (PUFA), and 12.5% Omefa-6 PUFA (Masa et al., 2011). Fresh water fish are known to be able to convert the essential fatty acids, including γ-linolenic acid (18:2n-6) and α-linolenic acid (18:3n-3), to longer chain and highly unsaturated fatty acids which have many health benefits (Kwetegyeka et al., 2008). Fatty fish and tropical freshwater fish, such as omena, are found to contain considerable amount Omega-3 PUFA (Masa et al., 2011).

Omega-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have significant health benefit for fetal brain and retina development, and also cell membrane function (Swanson, Block, & Mousa, 2012). DHA in the fetus is transferred from the mother through the placenta (Helland et al., 2008), thus mothers need to consume adequate amounts to support fetal development. The 2010 US Department of Health and Human Services suggested pregnant women should consume 8

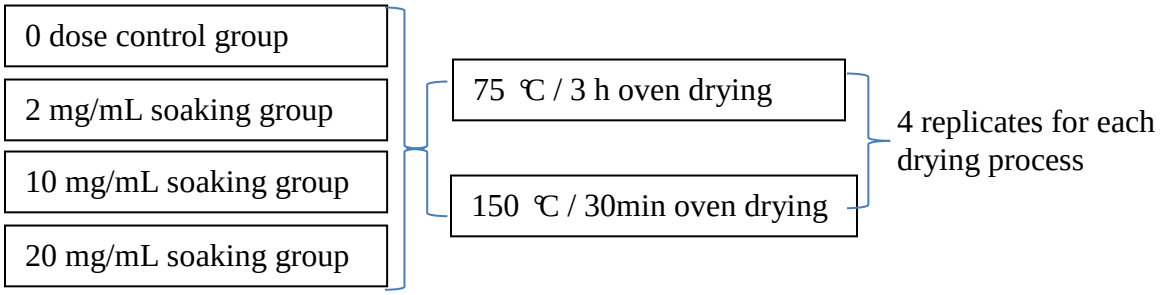
to 12 oz low mercury seafood per week (FDA, 2014), which equates to approximately 300 to 900 mg EPA combined DHA per day (Swanson et al., 2012).

The heating process may result in the damage of polyunsaturated fatty acids in fish, and also the development of rancid odor/ flavor. Natural antioxidants in spices and herbs have the ability to reduce the lipid oxidation, and also the off odor/ flavor generated by lipid oxidation in meat (J. I. Gray, Gomaa, & Buckley, 1996; Nissen, Byrne, Bertelsen, & Skibsted, 2004; Tomaino et al., 2005). The phenolic compounds in spices and herbs stabilize lipid peroxidation by acting as a radical scavenger and can also inhibit oxidizing enzymes (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009). Clove has been reported to have a relatively higher total phenolic content and antioxidant activity than many other spices (Shan et al., 2005). This study aimed to test the effectiveness of a clove-water extract as an antioxidant treatment to prevent lipid oxidation in omena fish during oven-drying process, by assessing the chemical indicators of lipid oxidation, the survival of long chain omega-3 fatty acids, and the sensory properties of the final products after processing.

## Material and Method

### Research Design

We studied the impact of two treatment conditions on lipid oxidation in oven-dried fish in a $4 \times 2$ complete experimental design with the following variables: dose of spice extract treatment and oven-drying time/temperature conditions.




◆ **Figure 8 4 × 2 complete experimental design**

## Selection of Treatment Conditions

Three doses of clove water extract were studied in this research to compare the antioxidant effects. Several studies have indicated that clove has the highest total equivalent antioxidant capacity among different spices (Katalinic et al., 2006; Shan et al., 2005). Importantly, it is also available and consumed in Kenya. Alcohol extraction, boiling water and distillation methods are widely used in spice antioxidant extraction. However, alcohol extraction and distillation methods are more expensive than water extraction and/or require the removal of solvent prior to use in food products for human consumption, which make them incompatible for use in small production kitchens in Kenya. Furthermore, a previous study showed that boiling the spices in water for 10 min can extract most of the water soluble antioxidants, and the antioxidants are stable during boiling for 30 min or longer (Arabshahi-D et al., 2007). Taken together, this suggests that boiling water extractions of clove are a suitable material for proof-of-concept testing.

Two temperature/time oven-drying conditions were used for drying fish. The low temperature (75 ℃) / long drying time (3 h) condition was designed to mimic the sun drying condition in Kenya, and to replicate conditions of a previous acceptance trial

(Gewa et al., 2014). A high temperature (150 ℃) / short time (30 min) condition was also used for drying the fish. These two temperature/time drying combinations were verified to result in a consistent weight, indicating the process removed all available water, and further time in the oven will not result in additional water loss. In this complete experimental design, all spice dose groups and the control group were put through both drying conditions. Lastly, each treatment condition (spice dose × oven-drying condition) was designed to have 4 replicates, to account for the variations caused by the oven-drying process.

## Materials and Chemicals

Approximately 8 kg of frozen whole omena samples and ground cloves were collected by Dr. Constance Gewa of the Department of Nutrition and Food Studies, George Mason University from a fishery in Kenya in August 2014. Omena samples were packaged approximately 1 kg per sealed bag, and transported on dry ice, $CO_2$(s) in a foam polystyrene cooler. Commercially ground clove samples from Nature's Own (Nairobi, Kenya) and mixed spices from Tropical Heat (Nairobi) were purchased from Kenya and transported at ambient temperature. Upon receipt, fish were stored in a -80 ℃ freezer until processing.

Reagents were obtained at ACS-grade, or higher, from the following companies.

Sigma-Aldrich (St. Louis, MO): 2N Folin-Ciocalteu reagent (FC Reagent), gallic acid, 2,2'azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchoman-2-carboxylic acid (Trolox), 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), Fluorescein, Butylated hydroxyanisole

(BHA), 2-Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Ammonium ferrous sulphate hexahydrate,

Fisher Scientific (Fair Lawn, NJ): Ultrapure water, Malonaldehyde bis (dimethyl acetal, TMP), $MnO_2$, FluoroNunc 96-well polystyrene plates, HPLC-grade methanol.

Acros Organics™ (Geel, Belgium): Sodium carbonate, Cumene hydroperoxide, Xylenol Orange

EMD Chemicals Inc. (Gibbstown, NJ): Sodium phosphate, dibasic, anhydrous; Sodium phosphate, monobasic, monohydrate.

## Sample Preparation

### Clove Water Extract

According to preliminary results, the total phenolic content increased with the dose of clove-water extract, and a dose of 10 mg/mL had the highest extraction efficiency and cost-effectiveness. Thus, three doses of clove extract were prepared. Ten mg/ mL was chosen as the primary study dose for reducing TBARS in oven-dried fish. One lower dose (2 mg/ mL) and one higher dose (20 g/ mL) were selected as comparative groups, with the highest and lowest doses being separated by a factor of 10; 2 g, 10 g and 20 g ground clove were weighed and added to 1 L boiling water to make 2 g/L, 10 g/L and 20 g/L clove water extracts. A spatula was used to mix the water and spice. The mixtures were kept simmering for 10 min and subsequently cooled down to room temperature in a 4 ℃ fridge. The mixtures were gravity filtered using Whatman Grade 1 qualitative filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA) to remove solid particles. Extracts were prepared daily.

For antioxidant capacity analysis of the clove extractions, 0.02 g, 0.1 g and 0.2 g ground clove were weighed into 15 mL glass test tubes; 10 mL ultrapure water was added to each tube, and caps were used to loosely cover the tubes. The tubes were put into a boiling water bath, brought to a boil, and kept simmering for 10 min. The tubes were removed from the water bath and cooled down to room temperature. The mixtures were gravity filtered using Whatman Grade 1 qualitative filter paper to remove solid particles. The filtrates were gathered in clean 15 mL centrifuge tubes and stored in 4 ℃ refrigerator.

**Oven-dried Omena**

Frozen omena was stored in -80 ℃ freezer and the 1 kg blocks were defrosted in a 0 to 5 ℃ refrigerator for 2 days before use. For each batch, 120 g of fish (wet weight) were weighed and blanched in boiling water for 15 s. The surface water was dried with tissue and the fish were soaked in cooled clove extract for 1 h at room temperature. The fish were removed from the soaking extracts and distributed in a single layer on a stainless steel wire mesh rack. The rack was put into the oven to dry according to the two treatment conditions: 150 ℃ × 30 min or 75 ℃ × 3 h. The racks were removed from the oven and cooled to room temperature. A coffee grinder was used to grind the dried fish samples into fine fish meal, which had a particle size able to pass through a 40-mesh sieve, with small pieces of dry ice (CO2(s)) mixed into the fish to maintain low temperature during the grinding. The ground fish samples were packaged in separate and double layered airtight bags with desiccant to prevent moisture accumulation. The samples were stored in -80℃ freezer until analysis.

Each treatment was prepared in four batches, and the treatment conditions and codes were reported in **Table 2**. Note that A0 is considered the control conditions, as it is the original conditions used during field testing of the nutritional product by Gewa et al. (2014).

**Table 2 Treatment Conditions and Codes***

| Drying Conditions | Clove water extract doses | | | |
|---|---|---|---|---|
| | 0 g/L | 2 g/L | 10 g/L | 20 g/L |
| 75 ℃ / 3h oven-dried | A0 | A2 | A10 | A20 |
| 150 ℃ / 30min oven-dried | B0 | B2 | B10 | B20 |

*Samples of different treatments are coded; letter A represents 75 ℃ / 3 h oven-drying condition; letter B represents 150 ℃ / 30 min oven-drying condition; the numbers represent the doses of clove-water extracts.

**Lipid Extraction**

The modified Folch method was used to extract the lipid from ground oven-dried omena. The procedure was previously described by Lin et al. (2004) and Ramalhosa et al. (2012). Extraction solvent was made by mixing ethyl acetate (EtOAc) and ethyl alcohol (EtOH) at 2:1 volume ratios. Two grams of ground fish sample was weighed into a 125 mL Erlenmeyer flask, and 20 mL of solvent was added at ratio 1:10 (w/v). The mixture was homogenized with a handheld homogenizer (Fisher Scientific, Waltham, MA) for 1 min and allowed to settle. Then, the mixture was gravity filtered with Whatman No.1 filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA). The filtrate and precipitate were collected. The precipitate was extracted for a second time using the same procedure. The filtrate was combined and added into a weighed 50 mL glass tube to evaporate the

solvent under a nitrogen stream. The tube was weighed, and sealed using Parafilm (Bemis, Oshkosh, WI) with nitrogen added after the evaporation. The tube with lipid sample was stored in 4 ℃ refrigerator for later use. All 32 samples were extracted and the yield was calculated.

## Chemical Analysis of Lipid Oxidation

### Modified Ferrous Oxidation-Xylenol Orange (FOX) Method

The peroxide value of each oven-dried fish sample was measured by using the modified xylenol orange method, which was previously described by Eymard & Genot (2003). Hydrogen peroxide was used as a standard (DeLong et al., 2002). To extract the peroxides, 30 mL of 4 ℃ HPLC-grade methanol was added to 3 g of ground fish sample (w:v, 1:10). The mixture was homogenized with a handheld homogenizer (Fisher Scientific, Waltham, MA) for 15 s, then centrifuged for 10 min at 7000 rpm and gravity filtered with Whatman Qualitative #1 filter paper (GE Healthcare Bio-Sciences, Pittsburgh, PA). The FOX reagent A was made by dissolving 0.25 mmol ammonium ferrous sulphate hexahydrate into 25 mmol 97% $H_2SO_4$, and the final volume was made up to 100 mL with water. To make the FOX reagent, 19 mg of xylenol orange was dissolved in 50 mL of HPLC-grade methanol, and then mixed into 25 mL reagent A, then the final volume of the FOX reagent was made up to 250 mL with HPLC-grade methanol. The FOX reagent contained 100 μM xylenol orange, 0.25 mM ammonium ferrous sulphate hexahydrate, and 25 mM $H_2SO_4$, respectively. The extract was diluted with methanol as needed to fit the standard curve. One-hundred-fifty μL dilutions and 1350 μL FOX reagent were vortex-mixed well, and were placed in the dark to react for 50 min at

room temperature. The absorption was read at 560 nm against the blank, which contained 150 µl methanol and 1350 µL FOX reagent. The standard curve contained 0 – 20 µM hydrogen peroxide. All 32 samples were tested and the reaction was performed in duplicates. Results are reported in µmol Hydrogen Peroxide Equivalent / g dried omena sample.

**TBARS**

The TBARS of each ground omena sample was determined according to a previously described lab protocol using 1,1,3,3-Tetramethoxypropane (TMP) as a standard (Salih et al., 1987). Two grams of ground omena was weighed into a 50 mL centrifuge tube and homogenized with 10 mL 20% TCA solution, 8 mL distilled water and 1.2 mL BHA ethanol solution using a homogenizer in ice bath for 60 s. After sitting for 1 h, the mixture was centrifuged at 2000 × g for 10 min; then, filtered; the filtrate contained secondary lipid oxidation products. The final reaction mixture contained 1 mL filtrate, 1 mL distilled water, and 2 mL TBA solution. The mixture was allowed to react in a 100 ℃ water bath for 20 min. After cooling to room temperature, the absorbance was measured at 532 nm. Results are reported in mg TMP equivalent/ g sample. This assay was performed in duplicate.

**Fatty Acid Composition**

Fish lipids are extracted from fish tissue using the modified Folch method above. The fatty acid composition was determined within two weeks. The extracted fish lipids were prepared for gas chromatography (GC) analysis by converting to fatty acid methyl

esters (FAMEs) according to a previously described procedure by Yu et al. (2002) with modification. In a fume hood, 0.2 mL toluene, 0.1 mL oil sample and 0.2 mL NaOH-MeOH (0.5 M) were mixed in a capped 15 mL glass test tube. The mixture was heated in a 60℃ water bath for 10 min. After cooling to room temperature, 0.5 mL HCl-MeOH (4%) was added to react for 5 min, then 3 mL distilled water was added to stop reaction. Three mL hexane was added, and the mixture was vortex mixed, and then centrifuged. A Shimadzu GC-2010 with a FID detector and a fused silica capillary column SPTM-2380 (30 m × 0.25 mm with a 0.25 μm film thickness) (Supelco Inc., Bellefonte, PA) was used. To identify each fatty acid, a GLC- 68D standard (Nu-Chek Prep Inc., Elysian, MN) was used for comparing retention time. The carrier gas Helium had a flow rate of 2.2 mL/min. Injection volume was 1 μL at a split ratio of 10/1. The oven temperature started at 142 °C and increased 6 ℃/ min to 184 ℃, then held for 3 min, and then increased by 6 ℃/min to 251 ℃. The FAMEs were quantified by calculating the percentage of area under each identified peak relative to the total area of the total area under all identified peaks. Results are reported in g FAME / 100 g total fatty acids. All 32 samples were analyzed.

## Chemical Analysis of Antioxidant Capacity

### Totol Phenolic Content (TPC)

The total phenolic content of each clove water extract sample was measured according to a previously described protocol in which gallic acid was used as a standard (Singleton, Orthofer, & Lamuela-Raventos, 1999). The standard curve was prepared in ultrapure water. The original extracts were diluted with ultrapure water at the ratio of 1:10. The final reaction mixture contained 50 μL sample, 250 μL Folin-Ciocalteu reagent,

and 3 mL of ultra-pure water, and was vortex-mixed for 5 s. After sitting for 5 min, 750 μL of 20% (w/v) sodium carbonate was added to start the reaction. After 2 h reaction at room temperature in the dark, absorbance was read at 765 nm. The reactions were conducted in duplicates, and results reported in μmol gallic acid equivalent (GAE) / L of clove water extract.

**Oxygen Radical Absorbance Capacity (ORAC)**

The ORAC of each clove water extract samples was determined using a procedure described by Ou et al. (2001) with fluorescein (FL) as a fluorescent probe (Whent, 2009); trolox (in 50% acetone) was used as a standard. The clove water extract samples were diluted with acetone to make 50% acetone solution. Other reagents were prepared in 75 mM pH 7.4 phosphate buffer. The samples were further diluted with 50% acetone until the absorption fell within the linear range of the standard curve. At the beginning of reaction, 225 μL fluorescein was combined with 30 μL of samples, standard, or solvent in a 96-well plate. The plate was covered and pre-heated at 37 ℃ for 20 min in the plate reader. Twenty-five μL of 0.36 M AAPH was immediately added to each well, and mixed with the tips. The plate was put back into the plate reader to take readings for 1.5 h. The results were reported as μmol Trolox equivalent / L extracts. Samples were tested in triplicate.

**ABTS•+ Radical Scavenging Assay**

The $ABTS^{\bullet}$+ radical scavenging assay was performed using a previously described lab protocol (Wojdyło, Oszmiański, & Czemerys, 2007) and trolox (with

acetone added to be in 50% acetone) was used as a standard. $ABTS^{\bullet+}$ stock solution was made by oxidizing ABTS stock with powdered $MnO_2$ to $A_{734\,nm} > 1.5$. The $ABTS^{\bullet+}$ working solution was made by dilute the stock with 1.5 mM phosphate buffer (PB) to $A_{734\,nm} = 0.7\ (\pm 0.02)$. The spectrophotometer was blanked with a mixture of 2 mL of 1.5 mM PB and 160 µL of ultrapure water. The final reaction mixture contained 2 mL $ABTS^{\bullet+}$ working solution and 160 µL sample (in 50% acetone), standard or blank (50% acetone), and was vortex-mixed for 30 s. The reaction took exactly 90 s and the absorption was measured at 734 nm. The results are corrected for assay dilutions and expressed in µmol Trolox equivalant / L extracts. Tests were in conducted duplicates.

**Sensory Evaluation**

Sensory analysis for fish cracker was used in this research to evaluate the impact of clove treatments on sensory attributes related to lipid oxidation. The sensory evaluation was exempted by the George Mason Institutional Review Board (IRB) for human research. As reported in other fish lipid oxidation studies, the PV and TBARS were both used as indicators of the extent of lipid oxidation (Liu, Zhang, & Wang, 2012; Pacheco-Aguilar, Lugo-Sánchez, & Robles-Burgueño, 2000); while, as described in former text, the peroxide value may decline in the later period of lipid oxidation due to the degradation of peroxides to form more stable secondary products, which are indicators of off-flavor and off-odor generated during lipid oxidation and are measured by the TBARS assay (Liu et al., 2012). Thus, the TBARS value is a better indicator for the sensory attributes of lipid oxidation. The chemically best fish sample (B10), which had the smallest TBARS value, was made into crackers for sensory evaluation. The

original fish cracker (A0, without spices treatment and oven-dried at 75 ℃ for 3 h) was used as the comparative control. The recipe was provided by Dr. Constance Gewa of the Department of Nutrition and Food Studies, George Mason University, and originally used in Kenya (**Appendix A**). The ingredients included wheat flour, ground omena, all-vegetable shortening, no added sugar apple sauce and sugar. A commercial mixed spice blend from Tropical Heat (Chirag Kenya Limited, Nairobi, Kenya), which contained cloves, ginger and cinnamon, was added to the mixed flour to disguise any flavor difference caused by the clove water extract during pre-treatment. Fish cracker samples were properly stored (covered by dark plates and put into air-tight bags) in the 0 ℃ refrigerator for one week and kept at room temperature for 30 min before being served in the evaluation session. Sensory tests were designed to evaluate the sensory quality differences between the two cracker samples. The sensory evaluation sheet is available in **Appendix B**. A triangle test and quantitative response scale tests were used. All sensory evaluation sessions were finished within two weeks.

Thirty untrained panelists were recruited from the student body of the College of Health and Human Services at George Mason University. For each sensory evaluation, a single fish cracker sample was displayed on a coded white paper plate. The sensory evaluation was carried out in a single nutrition classroom which was light and temperature controlled. Talking was not allowed during the evaluation session to prevent interpersonal influence. The triangle test was based on olfactory and gustatory parameters. Subjects were provided with one different and two same cracker samples and instructed to indicate the different sample. The participants were allowed to taste/smell back and

forth among all three samples until they had decided on the response. The significance of difference was measured by comparing with a table of minimum number of correct responses (ISO 4120:2004). In the quantitative response scale test, participants were required to rate two cracker samples one by one on a continuous 0 to 10 scale, the indicators included fishy odor, spicy odor, fishy taste, spicy taste, bitter taste, crispiness and stickiness; 0 indicated "least, not at all" and 10 indicated "most, extremely". Responses were assessed by measuring the distance from 0 on the scale where subjects indicated their rating. Subjects were also asked about their preference towards these two samples, their attitude towards eating fish, and the frequency of eating fish.

### Statistical Analysis

Data were analyzed using IBM SPSS (SPSS for Windows Version 22.0, 2013, IBM, Bethesda, MD). Data were reported in the format of mean ± standard deviation. Differences between means were determined using independent sample t-test or one-way analysis of variance (One-way ANOVA) with a Turkey's HSD Post Hoc test or a Games-Howell Post Hoc test, according to the equal or unequal variance of different groups. The sensory evaluation data was analyzed using a paired sample t-test. The correlations among means were determined using a two-tailed Pearson Correlation Coefficient; a p-value less than 0.05 was recognized as statistically significant.

## Result and Discussion

### Lipid Extraction

The crude fat content was determined using modified Folch method. The average crude fat contents of 75 ℃/ 3 h and 150 ℃/ 30 min oven-dried omena samples were 13.38% ± 3.57 (n = 4) and 13.38% ± 0.51 (n = 16), respectively (**Table 3**). The data

agreed with the previous report of 14.09% and 13.9% in dried omena (Bille & Shemkai, 2006 & Owaga et al., 2010). A large variation may due to a small sample size and incomplete extraction, and may also due to many other environmental factors that affect the fat content of omena fish, including seasons and food supplies (Kirema-Mukasa, 2012).

**Table 3 Water Loss and Crude Fat Content**

| | **75 ℃/ 3h oven-drying** | **150 ℃/ 30 min oven-drying** |
|---|---|---|
| Water loss (%, n = 3) | 83 ±1.62 | 83 ±1.52 |
| Crude Fat Content (%) | 13.38 ±3.57 (n = 4) | 13.38 ±0.51 (n = 16) |

## Chemical Analysis of Lipid Oxidation

### Peroxide Value

The modified ferrous oxidation-xylenol orange (FOX) method was used to measure the primary lipid oxidation product peroxides in oven-dried fish samples. The result (mean ±SD) was reported in **Table 4**. The peroxide value ranged from 0.034 to 0.196 μmol HPE/ g sample. The 150 ℃/ 30 min oven-dried samples had overall significantly lower peroxide values than that of the 75 ℃/ 3h oven-dried samples ($p<0.05$). For the same oven-drying condition, the peroxide value decreased when the clove dose increased. The sample A0 had the highest peroxide value and B20 had the lowest peroxide value. No statistical significance was found between samples B20 (0.034 ±0.002) and B10 (0.042 ±0.007), see **Figure 9**.

Lipid peroxidation is related to polyunsaturated fatty acids degradation. It indicates the initial stage of lipid oxidation, and a lower PV value may result from primary lipid oxidation product peroxides broken down to secondary products, which are measured by the TBARS assay (Melton, Black, Davis, & Backus, 1982; Ru ź et al., 2001). Thus, TBARS assay and PV assay are both needed to determine the extent of lipid oxidation.

**Table 4 Hydroperoxide value of oven-dried omena samples (µmol HPE/ g sample)**

| Drying Conditions | Clove water extract doses | | | |
|---|---|---|---|---|
| | 0 g/L | 2 g/L | 10 g/L | 20 g/L |
| 75 ℃ / 3h oven-dried | 0.196ef ±0.055 | 0.151de ±0.031 | 0.109cd ±0.027 | 0.092c ±0.020 |
| 150 ℃ / 30min oven-dried | 0.059b ±0.009 | 0.069bc ±0.021 | 0.042a ±0.007 | 0.034a ±0.002 |

* Values shown are mean ±standard deviation (n=4). HPE = hydrogen peroxide equivalent. Means marked with the same letter are not statistically significant ($p < 0.05$).

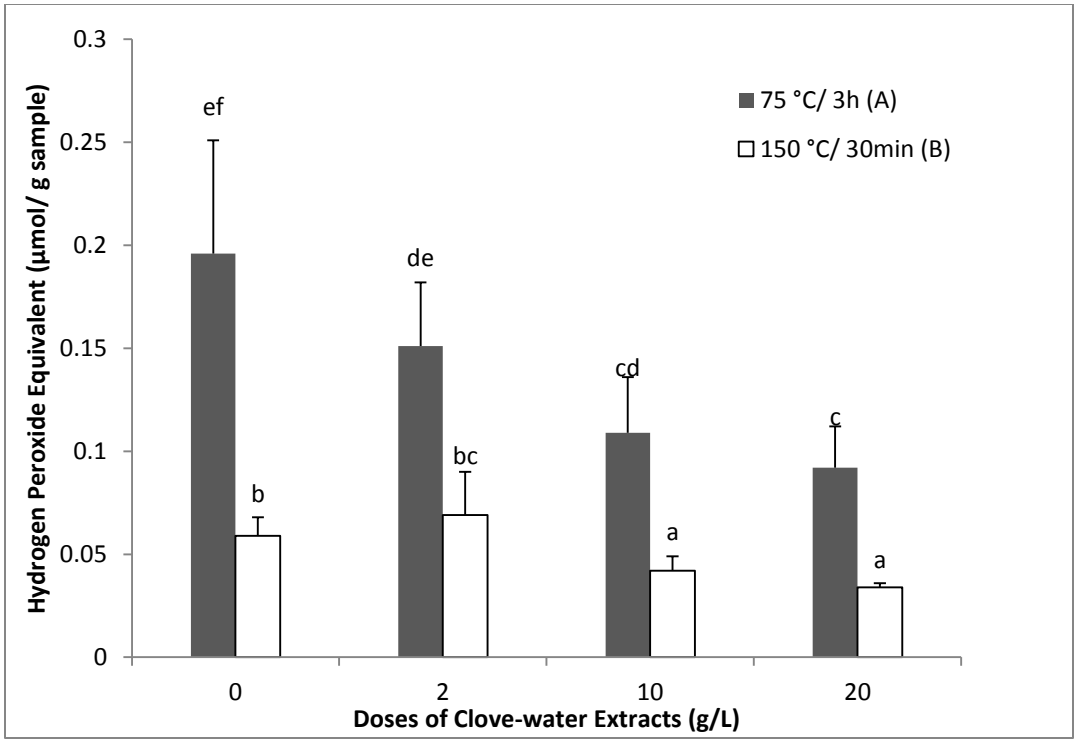



**Figure 9 Peroxide Value of Oven-dried Omena Samples**
Peroxide value is expressed as µmol hydrogen peroxide equivalent/ g sample. Vertical bars represent ± standard deviation (n = 4). Different letters represent significant difference ($P < 0.05$).

**Thiobarbituric Acid Reactive Substances (TBARS)**

The TBARS assay measured the secondary lipid oxidation products (MDA), which was generated during the oven-heating process. The tested fish samples were different in TBARS values, see **Table 5**. The result was reported in mean ± standard deviation (SD). There are significant differences in TMPE of the two drying methods, and the 150 ℃/ 30 min oven-drying procedure had significantly lower TBARS value than the 75 ℃/ 3 h oven-drying ($p < 0.05$). When under the same oven drying condition, the TBARS value decreased as the clove water extract doses increased. The sample A0 (without clove-water extract treatment and dried in 75 ℃ oven for 3 h), which was designed to imitate the local sun-dried omena products, had the highest TBARS value

(63.72 ±4.64 μg TMPE/ g sample). The sample B20, which was treated with 20 g/L clove water extract and dried in 150 ℃ oven for 30 min, had the lowest TBARS value of 11.18 ±1.39 μg TMPE/ g sample. These values correspond well to PV data. However, it was not significantly lower than that of sample B10 (10 g/L clove water extract treated), which was 14.53 ±2.49 μg TMPE/ g sample (p-value = 0.102), see **Figure 10**.

TBARS results are recognized to be directly related to change in flavor, color, odor, mouth texture, and nutrition benefits in fish or meat products (Owaga et al., 2009). A TBARS value lower than 6 μg (0.083 μmol) malondialdehyde (MDA) or 13.67 μg TMPE per g sample is regarded as acceptable in terms of fishy flavor and odor (Freeman & Hearnsberger, 1994). Sample A0 was used to imitate the original sun-dried omena in the Kenyan market. The rapid lipid oxidation is due to long low-level heating time and without antioxidant protection. Sample B10 and B20 both had significantly lower TBARS value than other samples, and B10 required half less clove in the sample preparation. Thus, for industry use, B10 is likely preferred.

When controlling for the dose of clove-water extract, the 75 ℃/ 3 h oven-drying condition had significantly higher TBARS value and PV value than the 150 ℃/ 30 min oven-drying condition. The significant difference may result from the time length that the food exposed to air and heat; also, a higher temperature oven-drying condition may rapidly remove the surface moisture and form a hard outer layer to protect the inner lipid from being oxidized. It can be predicted that, when removing the moisture from the fish, a higher temperature with a much shorter drying period can decrease the rate of lipid

oxidation, when comparing to a lower temperature with a longer drying period, even without adding antioxidants.

As previously observed in the PV result, the sample B10 and B20 had significantly lower peroxide values, when compared with other samples, suggested that the low PV were observed because they were less oxidized. The peroxide values agreed with the TBARS result, in which sample B20 and B10 generated significantly less primary product peroxides than other samples, and the lower peroxide value was not due to the breaking down of peroxides to secondary products. Comparing with A0, sample B10 had around 0.15 μmol less HPE/ g dried omena sample (**Table 4**). The result also suggested that 10 g/L clove-water extract soaking and 150 ℃/ 30 min oven-drying process can better protect unsaturated fatty acids in omena fish.

**Table 5 TBARS values for oven-dried fish samples (μg TMPE/ g sample)***

| Drying Conditions | Clove water extract doses | | | |
|---|---|---|---|---|
| | **0 g/L** | **2 g/L** | **10 g/L** | **20 g/L** |
| 75℃/ 3h oven-dried | 63.72ef ± 4.64 | 49.99de ± 10.86 | 38.77cd ± 6.62 | 31.97c ± 3.06 |
| 150℃/ 30min oven-dried | 20.85b ± 2.63 | 26.07bc ± 9.16 | 14.53a ± 2.49 | 11.18a ± 1.39 |

* Values shown are mean ± standard deviation (n=4). TMPE = 1,1,3,3-Tetramethoxypropane equivalent. Means marked with the same letter are not statistically significant ($p < 0.05$).

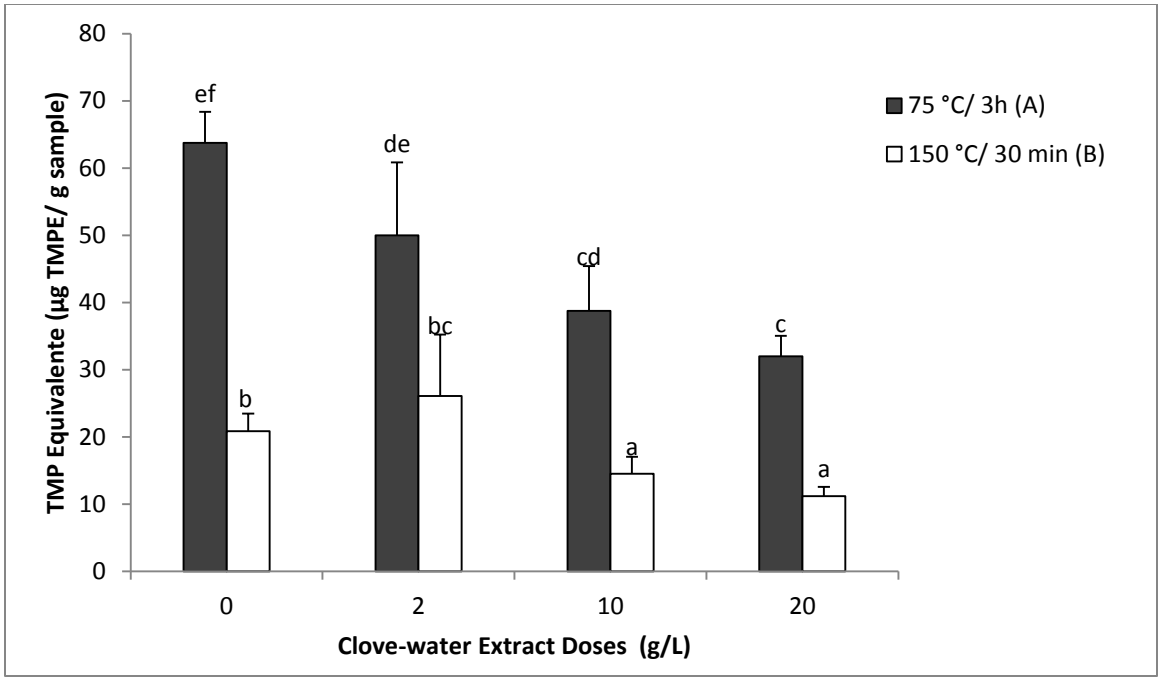



**Figure 10 TBARS value for oven-dried fish samples (µg TMPE/ g sample)**
*TBARS value is expressed as µg TMPE/ g sample. Vertical bars represent ± standard deviation (n = 4). Different letters represent significant difference ($P < 0.05$).

**Fatty acid composition**

The fatty acid compositions of all oven-dried omena samples were reported in **Table 6**. Palmitic (C16:0) was the most abundant fatty acid, which provided 25.56 to 28.16% of the total lipid content. The total amount of saturated fatty acids (SFA) in oil from oven-dried omena samples ranged from 38.06 to 42.11% of the total fatty acids. The proportion of total monounsaturated fatty acids (MUFA) in extracted oil ranged from 34.10 to 36.73%. The proportion of total polyunsaturated fatty acids (PUFA) ranged from 21.16 to 26.92%. Proportion of omega-3 and omega-6 polyunsaturated fatty acids ranged from 16.3 to 20.5% and 2.45 to 3.29%, respectively. The proportion of Docosahexaenoate (DHA, C22:6) and Eicosapentaenoate (EPA, C20:5) ranged from 5.04

to 8.6% and 5.19 to 6.79%, respectively. For 75 ℃/ 3h oven-dried samples, the proportions of Omega-3 and Omega-6 PUFA decreased with the dose of clove-water extract, see **Figure 11**. Sample B10 (10 g/L clove-water extract soaked and 150 ℃/ 30 min oven-dried) had the highest DHA content (8.60 ± 3.84 g/ 100 g oil). However, no significant difference was found among the means ($p > 0.05$). The proportions may have varied due to the different extent of lipid oxidation under processing conditions.

High Omega-3 PUFA content fish products are considered to have health benefits for pregnant women including reducing the risk of depressive symptoms after birth (Golding, Steer, Emmett, Davis, & Hibbeln, 2009) and improving the developmental birth outcomes (Hibbeln et al., 2007; Oken et al., 2008). Though not statistically significant ($p > 0.05$), under the 75 ℃ oven/ drying condition, the omega-3 and omega-6 PUFA may be better retained as the dose of clove-water extract/ the concentration of antioxidants increases, see **Figure 11.** A significant difference may appear when having a larger sample size. Sample B10 and B20 are preferred for retaining Omega-3 PUFA (21.15 g Omega-3 PUFA/ 100 g oil) during the oven-drying process, see **Table 6**. Even without a significant difference between treatments, these data demonstrate that the omena fish is a good source of long-chain omega-3 essential fatty acids.

According to TBARS and PV results, no significant difference was observed between B10 and B20 in terms of the extent of lipid oxidation and B10 requires half fewer cloves than B20. Thus, for industry uses, B10 is preferred.

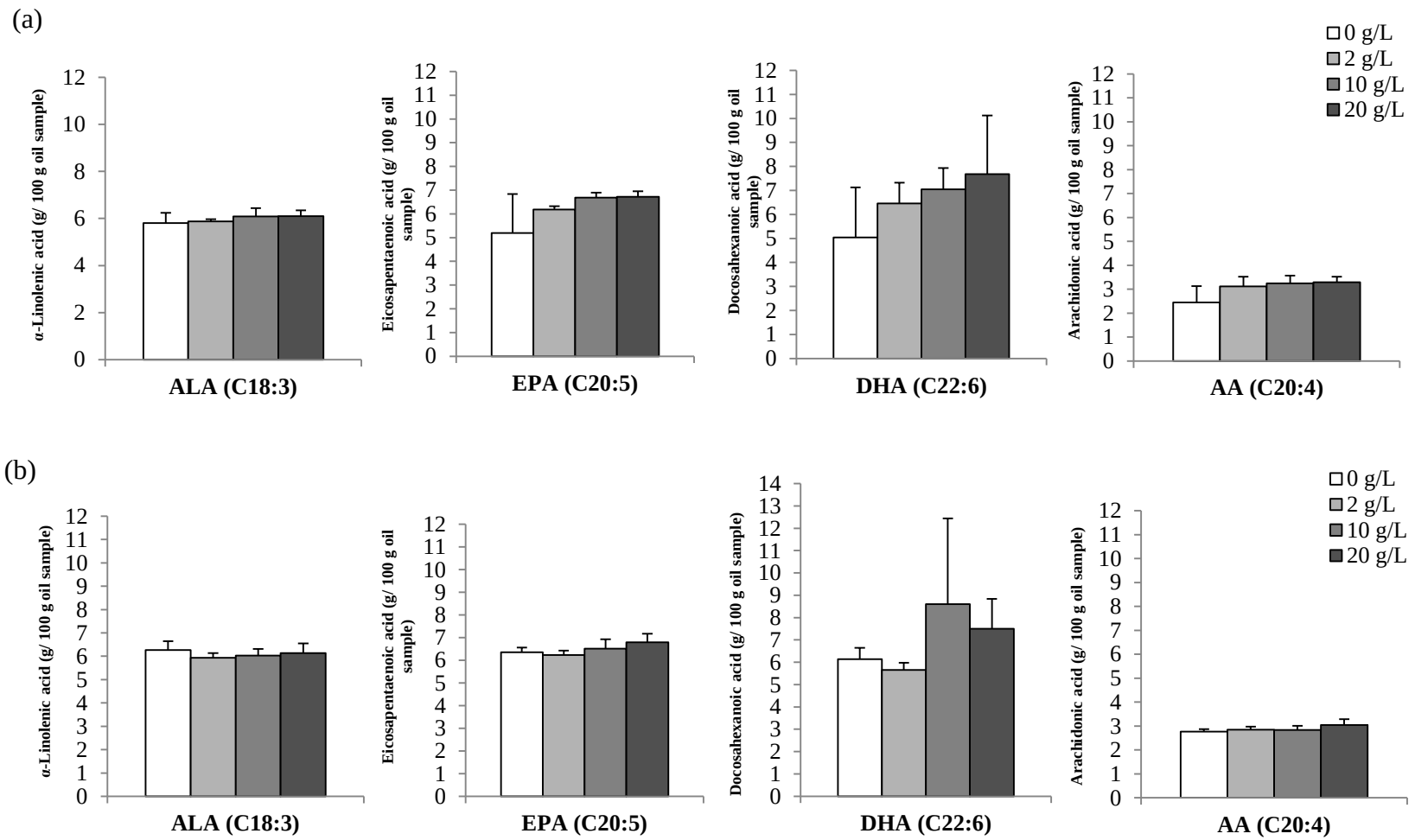



**Figure 11 Omega-3 and Omega-6 PUFA Content in Oven-dried Omena Samples**

*Fatty acid contents are expressed as g fattay acid/ 100 g sample. Vertical bars represent ± standard deviation (n = 4). Different letters represent significant difference ($p < 0.05$); (a) Oven-drying condition A: 75 ℃/ 3h; (b) Oven-drying condition B: 150 ℃/ 30min; ALA =α-Linolenic acid; EPA = Eicosapentaenoic acid; DHA = Docosahexanoic acid; AA = Arachidonic acid

**Table 6 Fatty Acid Composition of Oven-dried Omena Samples (g fatty acid/ 100 g oil sample)**

| | 75 ℃ / 3h | | | | 150 ℃ / 30 min | | | |
|---|---|---|---|---|---|---|---|---|
| | 0g/L | 2g/L | 10g/L | 20g/L | 0g/L | 2g/L | 10g/L | 20g/L |
| SFA | | | | | | | | |
| C14:0 | 3.54 ±0.31 | 3.42 ±0.08 | 3.36 ±0.09 | 3.28 ±0.09 | 3.35 ±0.19 | 3.11 ±0.41 | 3.03 ±0.26 | 2.95 ±0.61 |
| C16:0 | 28.16 ±1.49 | 27.13 ±0.91 | 26.57 ±0.55 | 26.04 ±0.65 | 25.87 ±0.55 | 26.64 ±0.60 | 25.56 ±1.61 | 25.89 ±0.64 |
| C18:0 | 10.41 ±0.40 | 9.99 ±0.55 | 9.85 ±2.62 | 9.80 ±0.37 | 9.52 ±0.27 | 9.94 ±0.16 | 9.47 ±0.56 | 9.70 ±0.33 |
| Total | 42.11 | 40.53 | 39.78 | 39.12 | 38.74 | 39.69 | 38.06 | 38.54 |
| MUFA | | | | | | | | |
| C16:1 | 12.35 ±2.92 | 11.85 ±2.42 | 11.72 ±2.29 | 11.52 ±2.37 | 14.20 ±0.08 | 14.24 ±0.42 | 13.86 ±0.96 | 12.78 ±2.15 |
| C18:1 | 24.38 ±1.22 | 23.02 ±0.98 | 22.38 ±0.86 | 22.60 ±1.02 | 22.42 ±0.46 | 22.42 ±1.61 | 21.16 ±0.79 | 22.10 ±1.05 |
| Total | 36.73 | 34.87 | 34.10 | 34.12 | 36.62 | 36.66 | 35.02 | 34.88 |
| PUFA | | | | | | | | |
| Omega-3 | | | | | | | | |
| C18:3 | 5.80 ±0.44 | 5.88 ±0.09 | 6.09 ±0.35 | 6.10 ±0.24 | 6.26 ±0.38 | 5.94 ±0.19 | 6.03 ±0.28 | 6.14 ±0.41 |
| C20:5 | 5.19 ±1.65 | 6.18 ±0.14 | 6.68 ±0.22 | 6.72 ±0.23 | 6.36 ±0.20 | 6.24 ±0.19 | 6.51 ±0.42 | 6.79 ±0.38 |
| C22:6 | 5.04 ±2.09 | 6.46 ±0.86 | 7.05 ±0.89 | 7.68 ±2.44 | 6.14 ±0.51 | 5.65 ±0.33 | 8.60 ±3.84 | 7.50 ±1.34 |
| Total | 16.03 | 18.52 | 19.83 | 20.50 | 18.79 | 17.84 | 21.15 | 20.43 |
| Omega-6 | | | | | | | | |
| C18:2 | 2.67 ±0.60 | 2.95 ±0.20 | 3.04 ±0.20 | 2.98 ±0.10 | 3.09 ±0.27 | 2.97 ±0.08 | 2.95 ±0.21 | 3.11 ±0.29 |
| C20:4 | 2.45 ±0.68 | 3.12 ±0.40 | 3.25 ±0.32 | 3.29 ±0.23 | 2.77 ±0.10 | 2.84 ±0.13 | 2.83 ±0.18 | 3.04 ±0.24 |
| Total | 5.12 | 6.07 | 6.29 | 6.27 | 5.86 | 5.81 | 5.78 | 6.15 |
| Total PUFA | 21.16 | 24.59 | 26.12 | 26.76 | 24.64 | 23.65 | 26.92 | 26.58 |

*Fish lipid was extracted from whole fish. Values are expressed in mean ± standard deviation (n = 4). SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. The fatty acids are reported as g/ 100 g oil sample.

## Chemical Analysis of Antioxidants

### Total Phenolic Content (TPC)

The clove water extracts analyzed in this study contained TPC levels between 4912.35 and 23970.54 μmol gallic acid equivalent (GAE)/ L clove-water extract (**Table 7**). As expected, the 20 g/L clove-water extract had significantly higher total phenolic content than that of the 2 g/L and 10 g/L ($p < 0.001$). The total phenolic content was in a significant positive correlation with the dose of clove-water extract, and the Pearson Correlation was 0.993 ($p < 0.001$).

As reported by Shan et al. (2005), the major phenolic compounds in clove are phenolic acids, flavonol glucosides, phenolic volatile oils and tannins; this is highly dependent on extraction conditions. Studies showed that clove antioxidant components are stable in boiling water for at least 30 min, and the lipid peroxidation inhibition capacity of cloves increased significantly (1.25 times) after being heated in 100 ℃ water for 30 min (Shobana & Akhilender Naidu, 2000). In this study, as previously stated, the clove-water extracts had total phenolic contents equated to approximately 20.39 – 41.78 g GAE/ 100 g ground clove, and it is higher than that of dried ground cloves (14.38 ± 0.006 g GAE/ 100 g clove (dw), (Shan et al., 2005)), which agreed with Shobana & Akhilender Naidu's (2000) study.

### Oxygen Radical Absorbance Capacity (ORAC)

The peroxyl radical scavenging capacity of clove-water extract was measured by the ORAC assay. The ORAC in tested clove-water extracts ranged from 4782.92 to

45146.92 μmol TE/ L clove-water extracts (**Table 7**). The 20 g/L clove-water extract had significantly higher ORAC than that of the 2 g/L and 10 g/L ($p < 0.001$). ORAC was in a significant positive correlation with the extract dose, and the two-tailed Pearson correlation was 0.985 ($p < 0.001$).

Previous study on spice antioxidants showed that clove extracted by acetone had an ORAC value of 1611.37 to 3084 μmol TE/ g clove (Dudonné et al., 2009; Wu et al., 2004). In this study, the boiling water extraction method extracted 2258.25 to 2391.46 μmol TE/ g dried ground clove, which agreed with the range.

#### ABTS•+ Radical Scavenging Assay

$ABTS^{\bullet+}$ radical scavenging assay was used to determine the total antioxidant capacity of the clove-water extracts. The $ABTS^{\bullet+}$ radical scavenging capacity of tested extracts ranged from 3126.25 to 25387.13 μmol TE/ L extracts. The radical scavenging capacity increased with the dose of clove-water extracts. Twenty g/L clove-water extract had significantly higher radical scavenging capacity than that of the 10 g/L and 2 g/L extracts ($p < 0.001$, **Table 7**). A significant positive correlation between extract dose and $ABTS^{\bullet+}$ radical scavenging capacity was detected with a two-tailed Pearson correlation 0.993 ($p < 0.001$).

The correlation between assays was examined (**Table 8**). Significant strong correlations were found between ABTS assay and ORAC assay (Pearson $R = 0.968$, $p < 0.001$), TPC and ORAC (Pearson $R = 0.982$, $p < 0.001$), TPC and ABTS (Pearson $R = 0.984$, $p < 0.001$).

The ORAC assay is based on hydrogen atom transfer, in which antioxidants and substrates compete for thermally generated peroxyl radicals; ABTS radical scavenging capacity assay and total phenolic content (TPC) assay are based on electron transfer, which measured the oxidant reduction capacity of antioxidants (Dudonné et al., 2009). Thus, three assays were used together to evaluate various modes of antioxidant activities. The clove-water extracts were used for soaking the omena fish to inhibit lipid oxidation; therefore, it is more important to study the antioxidants in the water extract than that of the whole spice. In this study, clove-water extract was found to have a considerable amount of antioxidants, and are stable during heating in boiling water, in agreement with previous studies (Dudonné et al., 2009; Shobana & Akhilender Naidu, 2000; Wu et al., 2004). Not enough data was obtained for the antioxidant stability during long-term storage, which requires further research.

**Table 7 Antioxidant Capacity of Different Doses of Clove-water Extracts**

| Doses of Clove Water Extracts | Assays | | |
|---|---|---|---|
| | TPC (µM GAE) | ABTS (µM TE) | ORAC (µM TE) |
| 2 g/L | 4912.35a ± 259.79 | 3126.25a ± 179.83 | 4782.92a ± 243.92 |
| 10 g/L | 14094.61b ± 494.21 | 14813.63b ± 387.41 | 21611.84b ± 5474.75 |
| 20 g/L | 23970.54c ± 1815.04 | 25387.13c ± 1236.19 | 45146.92c ± 2798.45 |

*Values shown are mean ± standard deviation (n = 3). Values in the same column that are represented by different letters are statistically different ($p < 0.05$). TPC = Total Phenolic Content, GAE = galic acid equivalent; TPC is expressed as µmol GAE/ 1 L extract; ABTS = ABTS cation radical (ABTS•+) scavenging capacity; ORAC = Oxygen Radical Absorbance Capacity; TE = trolox equivalent; ABTS and ORAC results are expressed as µmol Trolox equivalent/ 1 L extract.

**Table 8 Correlation coefficient (Pearson R) between Assays***

| | TPC | ABTS | ORAC |
|---|---|---|---|
| TPC | | | |
| ABTS | 0.984** | | |
| ORAC | 0.982** | 0.968** | |

*n = 3, values are Pearson R between assays, ** represents strong significance ($p < 0.001$)

The correlation between lipid oxidation in oven-dried fish and phenolic content of the clove water extracts was examined (**Figure. 12**). For 75 ℃/ 3 h oven-drying condition, a significant trend was observed that the TBARS value decreased as the total phenolic content increased. The 150 ℃/ 30min oven-drying condition resulted in relatively lower TBARS value when compared to 75 ℃/ 3 h oven-drying condition.

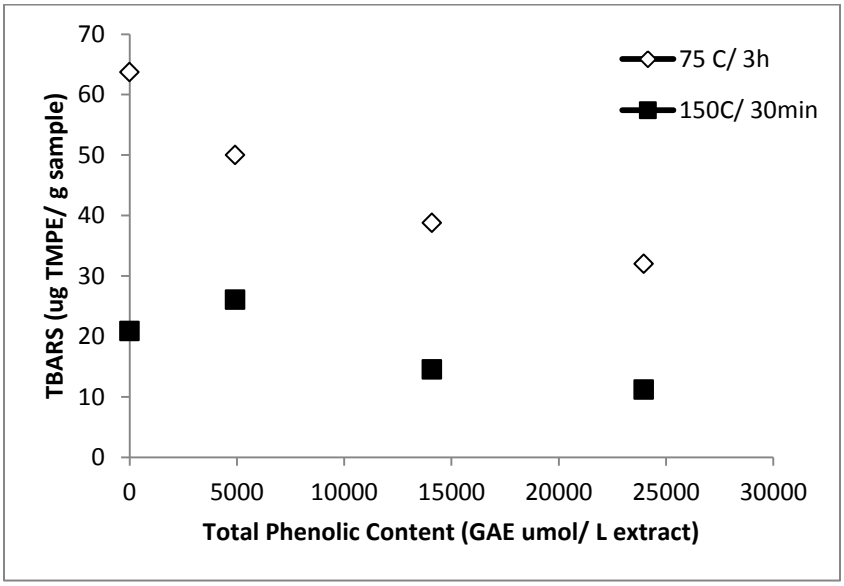



**Figure 12 Correlation between total phenolic content and TBARS value**

## Sensory Evaluation

Two samples were used to make fish crackers: A0 and B10. B10 was considered to be the chemically best sample. A triangle test was used for assessing the perception of differences in odor and taste between the fish crackers made from the B10 ground omena sample and the A0 ground omena sample. In the triangle test, 22 out of 30 (73.3%) participants correctly identified the different samples, suggesting significant differences in smell and taste existed between fish crackers made from clove treated fish sample (B10) and the original fish sample (A0), respectively ($p < 0.001$), according to the table provided in ISO4120: 2004.

The quantitative response scale test was used for rating the sensorial characteristics of odor, taste, and mouth texture of the fish cracker ($n = 30$). The result was shown in a radar map (**Figure 13**). The cracker made from B10 (10 g/L clove-water extracts treated and 150 ℃/ 30 min oven-dried omena) had significantly lower fishy odor score (3.24) and fishy taste score (2.89) than that of sample made from A0 (75 ℃/ 3h oven-dried omena), which had 5.26 for fishy odor and 5.20 for fishy taste (fishy odor: $p < 0.01$; fishy taste: $p < 0.001$). However, the B10 cracker (2.19) tasted significantly spicier than the A0 cracker (1.39) with a p-value less than 0.05, indicating that subjects recognized the presence of extra clove. The correlations between each characteristic were examined; significant correlations were found between fishy odor and fishy flavor (Pearson $r = 0.699$, $p < 0.001$), spicy odor and spicy taste (Pearson $r = 0.724$, $p < 0.001$), fishy taste and stickiness (Pearson $r = 0.506$, $p < 0.01$), bitter taste and stickiness (Pearson $r = 0.637$, $p < 0.001$).

In the preference test, 23 out of 30 (76.7%) subjects reported liking the B10 cracker sample better, 4 out of 30 (13.3%) subjects had no preference and 3 out of 30 (10%) preferred A0 cracker. Participants who preferred B10 cracker had an average score of 6.58 (between neutral and agree) in the extent of liking to eat fish, which was close to the score of subjects with no preference (6.38). Subjects who prefer the A0 cracker had a higher average score of 9.03, suggesting they may be less sensitive to fish flavor or generally enjoy the fish flavor; however, the differences were not statistically significant (ANOVA $p > 0.1$). The correlation between preference and how much they like to eat fish is not significant ($p > 0.1$). The frequencies of eating fish were reported in **Figure 14**. A significant positive correlation was found between the frequency of eating fish and how much they like to eat fish (Spearman's rho correlation coefficient = 0.585 and $p = 0.001$).

The sensory evaluation results agree with the chemical assay results; the cracker sample made from B10 had significantly lower fishy odor and fishy flavor than cracker sample made from A0, which agree with the TBARS data (sample B10 had a 52 μg TMPE/ g sample reduction when comparing with sample A0, see **Table 2**), even after the ground fish was made into fish crackers and reheated.

The approximate omega-3 fatty acids content in 150 ℃/ 30 min oven-dried fish is 21.15 g/ 100 g fish oil, and the crude fat content of oven-dried omena fish is around 13.38%; the fish flour accounts for 20.3% of the weight of whole cracker; thus the omega-3 PUFA in the fish cracker can be estimated by calculate the percentage weight

that every 100 g of omena fish cracker contains approximately 0.57 g of omega-3 fatty acids.

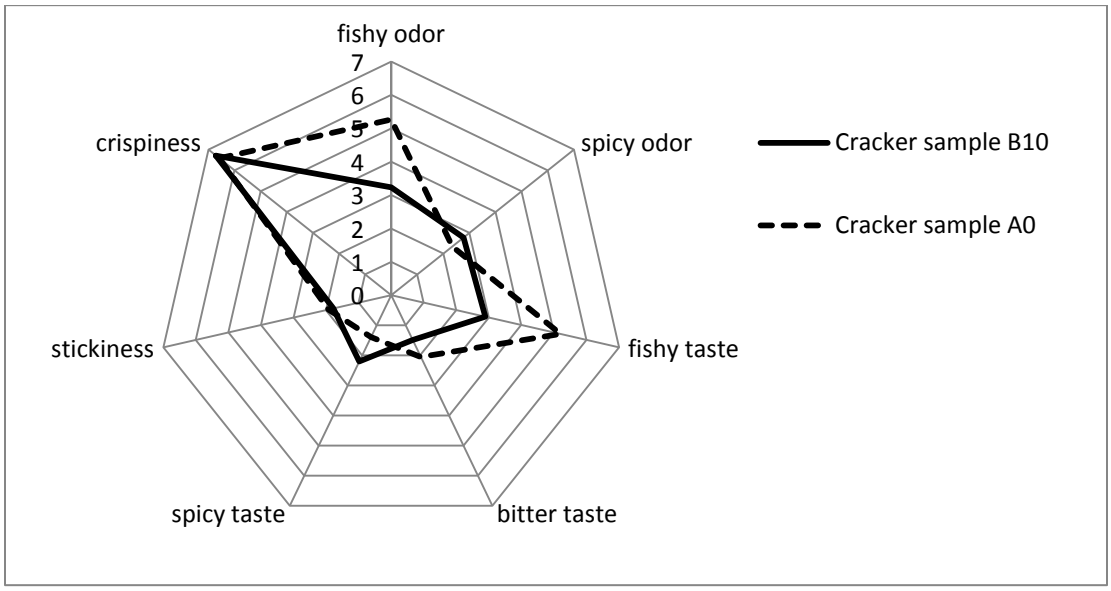



**Figure 13 Radar map of sensory characteristics of two omena crackers**
n = 30, scores range from 0 to 10, 0 indicates "least, not at all", 10 indicates "most, extremely"; data points are average score of 30 participants.

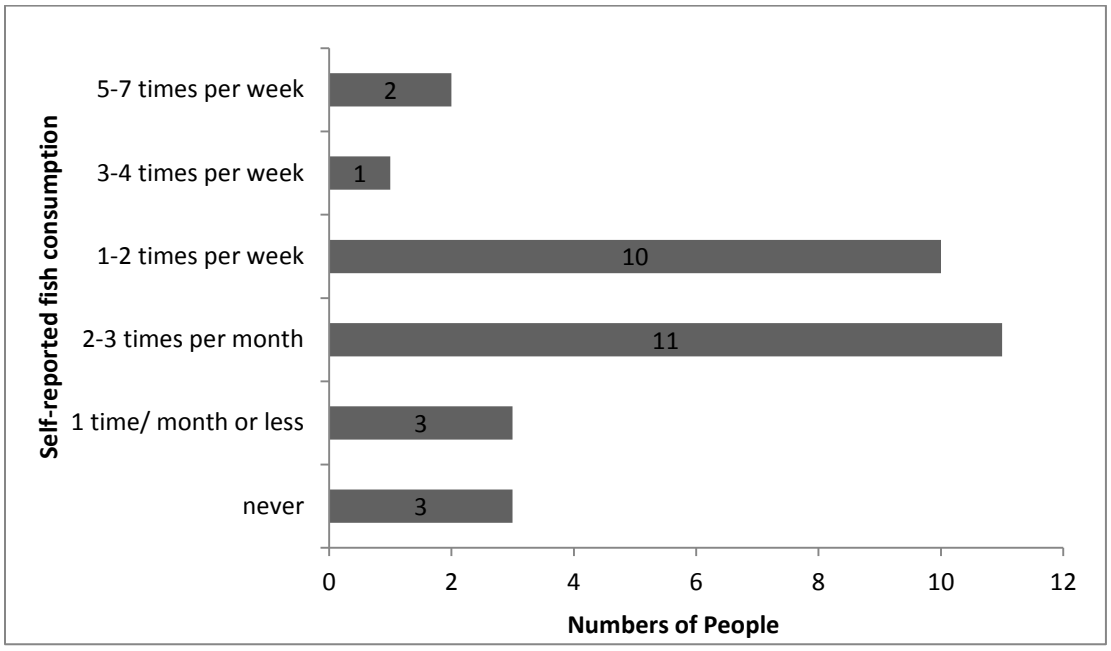



**Figure 14 Frequency of Eating fish**
n = 30, horizontal bars represent the number of participants in each category.

When comparing sample B0 with sample A0, B0 had significantly lower TBARS and PV values, which means by increasing the oven temperature and shorten the drying period can dramatically reduce the rate of lipid oxidation. However, B0 was not used in the sensory evaluation, because sample B10 was significantly less oxidized than B0. Future studies on the impact of different oven-drying conditions on sensory attributes of lipid-rich products may be needed.

As mentioned in the preparation of ground fish samples for chemical analysis, dry ice was added to maintain a low temperature when grinding the fish in a coffee grinder. The aim of adding dry ice is to protect the fish lipid from the extra heat conducted from the motor, which may influence the results of lipid oxidation assays. However, the dry ice

is not necessary when grinding the fish for making the cracker, in which a food processor or a mill is used.

The flavor and odor change during the storage of fresh fish was not studied. As previously stated, during frozen storage, trimethylamine-n-oxide (TMAO) can be reduced to dimethylamine (DMA) by an intrinsic enzyme, and fishy flavor was generated (Pedrosa-Menabrito & Regenstein, 1990). This enzyme reaction was found in certain gadoid species and the highest reaction rate was found at – 5 ℃ (Castell, Neal, & Dale, 1973). A study showed that the reaction was totally inhibited at a temperature of – 20 ℃ (Sotelo et al., 1995). Before preparing the oven-dried samples, the fresh omena fish was stored in a – 80 ℃ freezer, the growth of most microorganisms and enzyme activities were inhibited. Thus, it is not necessary to study the odor and flavor change during the frozen storage of omena fish.

This omena fish cracker uses locally available and affordable food resources in Kenya. And the clove is one of the most popular spices used in Kenyan dishes. To adapt laboratory based procedures to the field in Kenya, the pretreatment of omena fish and the oven-drying procedure are feasible. When preparing the clove-water extract, sieves and cloth can be used to remove large particles from the extract. Mill or large food processor can be used for making fish flour. There will be difference between laboratory result and industry production due to oven conditions, pretreatment conditions and the increased output; however, the improved omena fish cracker will still be better than the original cracker. The cost of making clove treated and oven-dried omena fish is definitely higher than making sun-dried omena fish; however, this improved drying procedure will result

in largely increased consumption of omena fish cracker and improved birth outcome, which will bring more life-long benefit than cost.

In this study, the improved omena fish cracker had significantly less fishy flavor and fishy odor than the original fish cracker. However, the limitation is that the sensory attributes and preference were not rated by the target population (pregnant women in Kenya). Thus, the flavor and smell of this cracker product may need further adjustment before entering the market.

Another limitation is on the shelf life and storage methods, which were not included in this study. The crackers were stored in a 0 ℃ refrigerator in air-tight bags and used for sensory analysis within one week. For future field or industry production, study on longer storage period and different storage conditions may be needed.

## Conclusion

This study indicates that using natural antioxidants from clove and high temperature (150 ℃), short time period (30 min) oven-drying may result in preferred dried omena fish products with lower oxidation level and less off-flavor and off-odor, and also better protect the omega-3 polyunsaturated fatty acids, especially EPA and DHA, in the fish as compared to the drying condition of low temperature (75 ℃) combined with long drying period (3h). Products made with fish prepared in this manner are likely to be better received and are recommended for future clinical trials in Kenya, particularly where subjects are asked to repeatedly consume the product. Additional research is needed to evaluate the shelf-life of the omena fish cracker products and the influence of storage conditions on lipid oxidation in the fish cracker samples.

# APPENDIX

## Appendix A: Fish Information and Fish Cracker Recipe

### Omena fish

Omena fish (*Rastreneobola argentea*) is a small fish (~2-3 inches) native to Lake Victoria, and is commonly consumed by the local inhabitants. Fresh omena was retrieved from the Lake Victoria fishery near Kisumu, Kenya, and immediately frozen and transported on dry ice by commercial airline to Fairfax. It has been stored in a commercial freezer upon receipt until present, and will remain so until approximately 48 h before preparation of crackers for sensory analysis. The fish is thawed in approximately 1 kg blocks in the refrigerator (between 32 - 40 ℉) for 48 h.

### Fish drying procedure

Ingredients

- 10 g Ground clove spice
- 200 g Omena fish
- Tap water

Instructions

1. Prepare clove water extract.
   a) In a pot with lid, bring 1 liter tap water to boil;
   b) Add 10 g ground clove into the boiling water, keep the lid on and simmer for 10 min; Cool the clove-water mixture to room temperature;
   c) Filter the clove-water mixture to remove the ground clove particles.
2. Pre-treat and dry the fish.
   Thaw the fish thoroughly in 0℃ refrigerator.

   For the clove treated fish crackers:
   a) Preheat the convection oven to 150 °C;
   b) Blanch the fish in boiling water for 15 s;
   c) Soak them in clove water extract for 1 hour (room temperature);
   d) Lay the fish onto a wire rack and put into the preheated convection oven and bake them for 30 min;

   For the original fish crackers:
   a) Preheat the convection oven to 75 °C;

b) Blanch the fish in boiling water for 15 s;
c) Lay the fish onto a wire rack and put into the preheated convection oven and bake them for 3 h;

3. Cool the fish to room temperature and grind the fish to fish flour with a coffee grinder.

**Fish cracker recipe**
1 serving (makes 7 six-cm wide, half-cm thick cookies)

Ingredients

- 31g Dry Fish flour
- 46g Self-Rising Wheat Flour
- 19g Sugar
- 19g Applesauce (no added sugar variety)
- 38g Vegetable shortening (like Crisco)
- Pinch of salt

Instructions

1. Pre-heat the oven to 350 degrees Fahrenheit (350 °F)
2. Cream sugar and shortening until smooth
3. Stir in the apple sauce
4. Mix the flours and salt in a separate bowl
5. Add the flour mixture into the sugar-shortening mix and work into a dough
6. Roll out dough into a six-cm wide cylindrical shape. Slice dough into half-cm thick slices. This should give you about 7 slices per serving.
7. Place the slices onto a baking tray.
8. Bake at 350 °F for about 13 min or until the edges turn brown.
9. Once cooked, let stand for a minute then remove and place on a cooling rack.

## Appendix B: Sensory Evaluation Form

Please do not write your name.

### Sensory Evaluations of Fish Cracker – Part 1

**Description:** You will be provided three fish cracker samples. Two of them are the same product, and one is different.

**Instructions:**

1. Please write down the sample codes of three samples you have received, in the table below.
2. Taste each sample, with the intent of determining which sample is different from the other two samples. Water and saltine crackers are available for you to rinse your mouth in between each sample.
3. Then, when you have made a decision, circle the sample in the table that you assume is different from other two samples.

| | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| **Code:** | | | |

When you are finished, do not turn the page. Please wait for further instruction.

## Sensory Evaluations of Fish Cracker – Part 2

**Description:** In this section, you will be provided with two cracker samples and asked to provide your opinion about the sensory attributes of the product.

**Instructions:**

1. Please answer this first question by circling your response:

   **Are you *able* to recognize/identify a "fishy" flavor? Yes No**

2. Please write the sample code of your first sample on the line provided at the top of the next page.
3. First, smell the provided fish cracker sample, then provide ratings from 0 – 10 for A1 and A2. (A score of "0" indicates "least, not at all" and "10" indicates "most, strongest".)
   a. ***To indicate your rating, draw a single vertical line on the scale.***
4. Then, taste the food and provide ratings for B1, B2, and B3.
5. Lastly, taste the food and provide ratings for texture in C1 and C2. Rinse your mouth with water and saltine crackers as needed.

6. When finished with your first sample, raise your hand to receive your second sample.
7. Repeat steps 1-5 with your second sample.

8. When finished with your second sample, please proceed to Part 3 on the final page.

Please do not write your name.

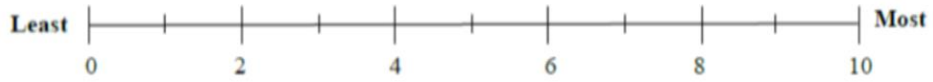
**Part 2 cont'd.**

**First Sample Code:** _____

**A. Odor. Based on the smell, please rate:**

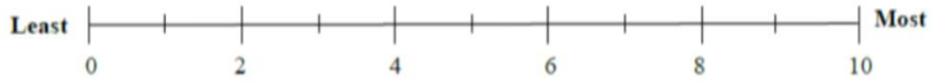
Intensity of fishiness *odor*


Intensity of spiciness *odor*

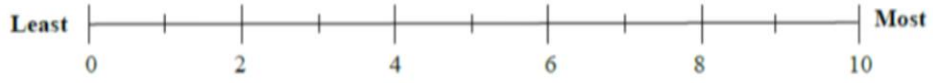



**B. Taste. Based on the taste, please rate:**

Intensity of fishiness *taste*




Intensity of bitterness

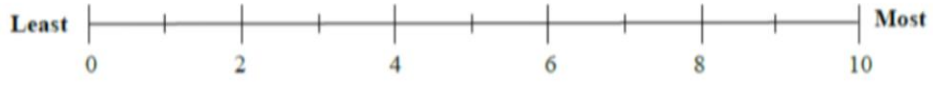



Intensity of spiciness *taste*


**C. Mouth texture. Based on the texture, please rate:**

Intensity of stickiness


Intensity of crispness




When finished, please raise your hand to receive your second sample.

3

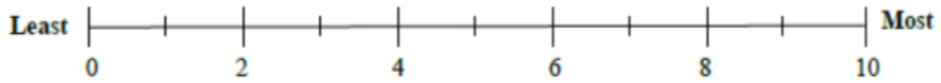
Please do not write your name.

**Part 2 cont'd.**

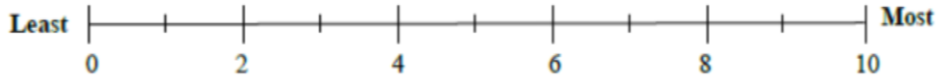
**Second Sample Code:** _____

**A. Odor. Based on the smell, please rate:**

Intensity of fishiness *odor*

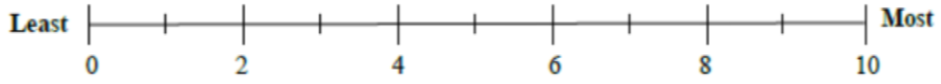



Intensity of spiciness *odor*

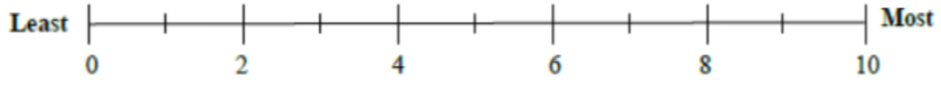



**B. Taste. Based on the taste, please rate:**

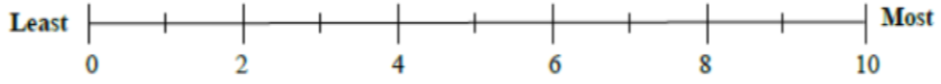
Intensity of fishiness *taste*




Intensity of bitterness

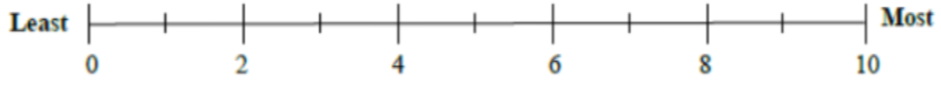



Intensity of spiciness *taste*

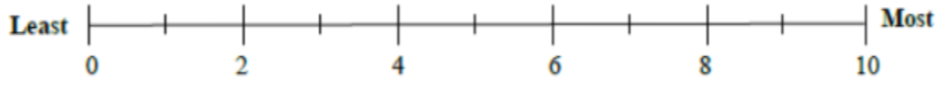



**C. Mouth texture. Based on the texture, please rate:**

Intensity of stickiness




Intensity of crispness




When finished, please proceed to Part 3 on the next page.

Please do not write your name.

## Sensory Evaluations – Part 3

**Description:** We'd like to ask a few final questions.

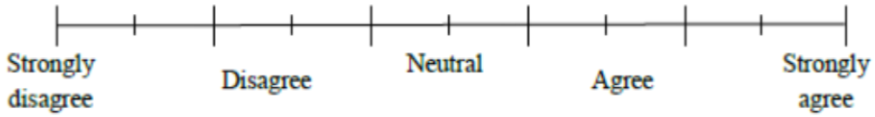
1. Do you have a preference for one of the samples in Part B? (Please circle.)

**Yes** **No**

**If "Yes", please write the code for the sample you preferred: _____**

2. Indicate the extent to which you agree with the following statement:

*"In general, I like to eat fish."*




3. On average, how often do you eat fish? (Please circle.)

a. Never
b. 1 time per month or less
c. 2-3 times per month
d. 1-2 times per week
e. 3-4 times per week
f. 5-7 times per week
g. More than 7 times per week

**Thank you for your time and taste!**
**Please return this survey to the research team.**

5

## Appendix C: IRB Approval Letter


Office of Research Integrity and Assurance

Research Hall, 4400 University Drive, MS 6D5, Fairfax, Virginia 22030
Phone: 703-993-5445; Fax: 703-993-9590

| | |
|---|---|
| DATE: | May 28, 2015 |
| TO: | Margaret Slavin |
| FROM: | George Mason University IRB |
| Project Title: | [682849-2] Sensory Test: SPICES TO PREVENT LIPID OXIDATION AND IMPROVE ACCEPTABILITY IN OVEN-DRIED FISH |
| SUBMISSION TYPE: | Amendment/Modification |
| ACTION: | DETERMINATION OF EXEMPT STATUS |
| DECISION DATE: | May 28, 2015 |
| REVIEW CATEGORY: | Exemption category #6 |

Thank you for your submission of Amendment/Modification materials for this project. The Office of Research Integrity & Assurance (ORIA) has determined this project is EXEMPT FROM IRB REVIEW according to federal regulations.

Please remember that all research must be conducted as described in the submitted materials.

Please note that any revision to previously approved materials must be submitted to the ORIA prior to initiation. Please use the appropriate revision forms for this procedure.

If you have any questions, please contact Bess Dieffenbach at 703-993-5593 or edieffen@gmu.edu. Please include your project title and reference number in all correspondence with this committee.

This letter has been electronically signed in accordance with all applicable regulations, and a copy is retained within George Mason University IRB's records.

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