HISTAMINE LEVELS IN FROZEN AND SMOKED FISH IN NUNGUA MARKET

BY

ARABA INCOOM

B.Sc. FOOD SCIENCE AND TECHNOLOGY (HONS)

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DECLARATION

I HEREBY DECLARE THAT I HAVE WHOLLY UNDERTAKEN THE STUDY REPORTED HEREIN UNDER THE SUPERVISION OF DR. FRANCIS ALEMAWOR AND THAT EXCEPT WHERE REFERENCES HAVE BEEN DULY CITED, THIS DISSERTATION IS THE OUTCOME OF MY RESEARCH. NEITHER ALL NOR PARTS OF THIS THESIS HAVE BEEN PRESENTED FOR ANOTHER DEGREE ELSEWHERE.

ARABA INCOOM _____ _____
(M. SC. STUDENT) SIGNATURE DATE

FRANCIS ALEMAWOR, PhD _____ _____
(SUPERVISOR) SIGNATURE DATE

PROF MRS I. ODURO _____ _____
(HEAD OF DEPARTMENT) SIGNATURE DATE

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LIST OF ABBREVIATION

ANOVA	Analysis of Variance
^{14}C	Carbon 14
$(CH_3)_2S$	Dimethylsulphide
CH_3SH	Methylmercarptan
EF	Extraction Factor
FAO	Food and Agricultural Organization
FDA	Food and Drugs Administration
H_2S	Hydrogen Sulphide
HPLC	High Performance Liquid Chromatography

HX	Hypoxanthine
NH_3	Ammonia
OPA	Ortho – Phthaldialdehye
PCR	Polymerase Chain Reaction
TMA	Trimethylamine
WHO	World Health Organization

ABSTRACT

Histamine is a biogenic amine produced in fish tissue through the decarboxylation of free histidine by exogenous decarboxylases released by microorganisms. Consuming fish contaminated by histamine beyond certain levels may result in histamine (or scombroid) poisoning characterized by allergies and other related seafood illnesses. However, survey studies assessing histamine levels of fish on local markets which could serve as baseline data for evaluating the related risk of exposure to seafood illnesses particularly in developing countries like Ghana are lacking. The findings of this project seek to create awareness of histamine levels in frozen and smoked fish on the Ghanaian (Nungua) market, especially under situations where storage conditions may be suboptimal such as when energy supply is limited. Two (2) species of fish namely; Atlantic horse mackerel (commonly known as Kpanla) and Mackerel (popularly known as Salmon on the Ghanaian market) were purchased from cold stores in the Nungua market. Smoked fish samples were also purchased from five (5) different sellers. Histamine was extracted from fish into perchloric acid solution after homogenization and analyzed using high performance liquid chromatographic method with ultra violet detector. The levels of histamine were lower in frozen Atlantic mackerel and it ranged from 0 - 1.67 mg/kg and the concentrations of Atlantic horse mackerel ranged from 0 - 25 mg/kg. However, both did not exceed the critical limit of 100 mg/kg for frozen fish. Histamine levels in smoked Atlantic fish ranged from 13-27 mg/kg and concentrations of smoked Atlantic horse mackerel also ranged from 12-33 mg/kg. However, the histamine levels measured in the samples did not exceed the critical limit of 200 mg/kg for smoked fish. In conclusion, the study found histamine levels in the selected species on the Nungua market to be within the internationally acceptable limits suggesting they were safe for consumption in relation to histamine concentrations.

CHAPTER ONE

1 INTRODUCTION

1.1 Background

Fish is a common source of high quality protein and contributes about 16 % of animal protein consumed by the world's population (FAO, 1997). Due to its high level of protein, it is susceptible to spoilage if not stored under right temperature conditions. Fish spoilage may occur through autolytic changes which involve self digestion by enzymes; oxidation and hydrolysis reactions and microbial action mediated by microbes such as *Morganella morganii*, *Enterobacter agglomerans*, *Enterobacter intermedium* etc. Microbial spoilage produces histamine ($C_5H_9N_3$) which is a foodborne toxin that is typically associated with fish species belonging to the scombridae and scomberesocidae families (scombroid fish). These include tuna, mackerel, herring, sardines, bonito etc.(Clark *et al.*, 1999).

Scombroid fish is highly rich in free histidine which when acted upon by the enzyme histidine decarboxylase (found in contaminating bacteria) is converted to histamine (Taylor *et al.*, 1989). This biogenic amine may be produced as a result of poor fish handling processes and storage. This constitutes the major component of scombrotoxin, although other compounds such as putrescine and cadavarine may also play a role. The risk of histamine/scombroitoxin poisoning is greatly increased when spoiled fish is consumed (Clark *et al.*, 1999).

Scombroid food poisoning is characterized by facial flushing/sweating, burning-peppery taste sensations in the mouth and throat, dizziness, nausea, headache, tachycardia, cold-like symptoms.

Other symptoms include facial rash and torso or body rash, short-term diarrhea, and abdominal cramps as well as occasional severe symptoms like blurred vision, respiratory distress and swelling of the tongue (Taylor *et al.*, 1989).

Histamine formation in fish is both temperature and time dependent and immediate freezing or cooling below 4 degrees Celsius is necessary to avoid histamine accumulation above acceptable levels (typically 100 mg/kg) (European Commission Regulation, 2005).

In instances where cold chain is poorly maintained and proper hygienic conditions are lacking such as may pertain in smaller coastal vessels or during inconsistent energy supply in cold stores, histamine development can be accelerated.

1.2 Problem Statement

Seafood constitutes a major component of the diet of most populations, thus illnesses associated with their consumption are of utmost public health concern globally. High histamine levels may be associated with seafood related illnesses such as tachycardia, short-term diarrhea and abdominal cramps. An important cause of allergic reactions associated with seafood consumption is histamine intoxication which occurs when fish is poorly refrigerated (Gonzaga *et al.*, 2009) or processed. However, survey studies assessing histamine levels of fish on local markets which could serve as baseline for evaluating the related risk of exposure to seafood illnesses particularly in developing countries are lacking.

In instances where energy backup is not available or inadequate, the cold chain of frozen items in cold stores may be altered, creating favorable conditions for the buildup of histamine in fish products.

1.3 Justification

In Ghana, some scombroid fish species are very common, most of which are imported or brought from high seas. The findings of this project seek to create awareness of histamine levels in frozen and smoked fish on the Ghanaian market, especially under situations where storage conditions may be sub-optimal such as when energy supply is limited.

1.4 Overall Objective

To assess the levels of histamine contamination in frozen and smoked fish sold on the local market.

1.4.1 Specific Objective

- To determine histamine levels in frozen and smoked scombroid fish from Nungua. market.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Spoilage of fish

Fish is considered spoilt when undesirable post-harvest changes occur in its original properties (Datta Munshi *et al.*, 2010). Conversely, fish freshness may be defined as fish with all the original features unchanged (Getu *et al*., 2015). Spoilage may occur within a short period of time if fish is not stored under the appropriate temperature conditions. Due to its high content of moisture, fats, free amino acids, nitrogenous compounds and digestible proteins, fish is highly perishable. Freshness of fish is a major determinant of its safety for consumption and market value. The beginning of fish spoilage is characterized by loss of its characteristic odour and taste due to autolytic degradation. The final stages of spoilage are marked by either tissue softening (or toughening) and the production of volatile unpleasant-smelling odours as a result of the metabolic activities of microbes (Fraser and Sumar, 1998). Features that may be indicative of fish spoilage include changes in the overall colour, texture, odour, softness/toughness of muscles and the colour of gills (Datta Munshi *et al.*, 2010). Maintaining the fresh qualities of fish and fish products are difficult mainly because the rich nutrient environment in fish supports the rapid multiplication of diverse microorganisms that mediate spoilage. Traditionally, fish in the wild are caught with nets, or lines with baits attached hooks. It is therefore difficult to often control the initial quality of freshly caught fish with any degree of repeatability prior to storage. The proximity of fish farms to suitable storage facilities and the timeliness in harvesting has to some extent contributed to reducing the variability of fresh fish quality. The activities of endogenous enzymes and bacterial growth that contribute to fish spoilage are enhanced by stresses and mechanical damage associated

with capture, the structure and composition of fish, pH changes, and temperature prior to storage *(*Jeyasanta *et al,* 2015*)*.

2.2 Microbial flora in fresh fish

The microbial flora of fresh fish is dependent of the type of fish species, the habitat and the method of sampling employed in their determination. The psychrotropic Gram-negative genera are the most common among the bacterial flora of cold water fish (Shewan, 1977) Microorganisms in this genera include; *Acinetobacter, Flavobacterium, Moraxella, Shewanella* and *Pseudomonas.* In addition, the flora may also include Gram-positive organisms like *Bacillus, Micrococcus, Clostridium, Lactobasillus* and coryneforms (Huss, 1995).

Fish from tropical waters have been reported to mainly harbor Gram-positive *Bacillus* and *Micro coccus* (Shewan, 1977). However, later reports suggested that while Gram-positive and enteric bacteria may dominate the micro flora of fish from tropical waters, the overall flora is usually not very different from those found in fish from temperate waters (Huss, 1995). Thus, there are a wide range and number of microorganisms on fish. These microorganisms contribute in varied proportions to fish spoilage depending on their growth rate, however, a number of microorganisms on fish do not play any significant role in spoilage (Huss, 1995).

High microbial load in fish may not always correlate to spoilage (Alur *et al.*, 1971). It has been reported that mackerel homogenate containing 10^7 -10^8cells/ml of *Micrococcus colpogenes* did not show any indication of spoilage (Alur *et al.*, 1989).

Therefore, it is erroneous to assume that the most predominant microorganisms found in fish are the cause of spoilage. Spoilage flora and spoilage bacteria should be distinctively identified and not confused with each other. The bacteria present on spoiled fish is referred to as spoilage flora

whiles the bacteria which is responsible for producing compounds (Table 1) resulting in offodours and off-flavours in spoilt fish is referred to as spoilage bacteria (Huss, 1995). Autolytic enzymes naturally present in fish also produce spoilage compounds.

Table 1. Bacterial spoilage compounds

Specific spoilage bacteria	Spoilage compounds
Shewanella putrifaciens	TMA, H_2S, CH_3SH, $(CH_3)_2S$, HX
Pseudomonas spp.	Ketones, aldehydes, esters, non-H_2S sulphides
Photobacterium phosphoreum	TMA, HX
Vibrionacaea	TMA, H_2S
Aerobic spoilers	NH_3, acetic, butyric and propionic acid

Source: Church, 1998. Note: TMA = trimethylamine, H_2S = hydrogen sulphide, CH_3SH =methylmercarptan, $(CH_3)_2S$ = dimethylsulphide, HX = hypoxanthine, NH_3= ammonia

2.3 Bacterial deterioration of fish

When a fish dies, the defensive systems which prevent attack of tissues by bacteria stop functioning. This allows the multiplication of inherent bacteria and invasion of exogenous bacteria via the skin, into the body cavity and intestine which spreads to the gills and the vascular system. Bacterial growth is promoted through rich nutrients which are as a result of low molecular weight compounds and soluble proteins produced from the body of fish during autolysis after rigor mortis.

Even at low temperatures, a variety of proteases and other hydrolytic enzymes secreted by invading bacteria can work on fish muscle to cause deterioration (Venugopal, 1990). Factors that affect microbial contamination and growth comprise of fish species and size, catch technique, on-board treatment/handling, sanitation on fishing vessel, storage conditions and processing (Chen and Chai, 1982, Ward and Baj, 1988). If handling and storage conditions are not optimal, the rate of microbial contamination and growth is increased.

2.4 Histamine production in fish

Histamine is a biogenic amine produced in fish tissue through the decarboxylation of free histidine by exogenous decarboxylases released by microorganisms. Different bacteria species including both Gram positive and Gram negative bacteria can facilitate histamine formation in fish (Ladero *et al.*, 2010). Histamine levels increase as fish decomposes, however, it is rarely found in fresh fish (Shakila *et al.*, 2003).

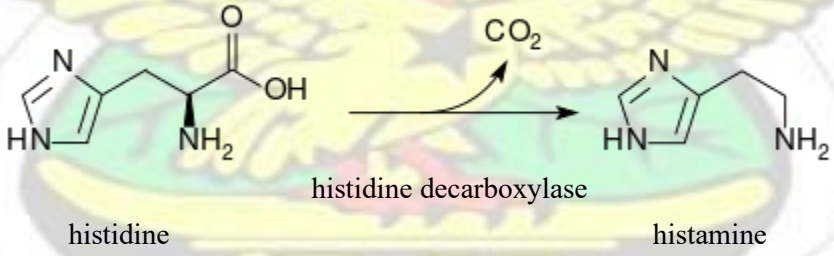


Figure 1. Conversion of histidine to histamine by histidine decarboxylase

At high abuse temperatures, histamine formation is hastened due to a more rapid multiplication of histamine producing bacteria. However, histamine can be produced at or near refrigeration

temperature so far as the histidine decarboxylase has been formed. The enzyme remains stable in a wide range of temperature, example it can be reactivated after thawing.

Histamine production can be discontinued at a temperature of -18 ºC or below and continued at a higher rate at abusive temperatures such as 21.1 ºC or higher (FDA, 2011). Histamine which has already been formed cannot be eliminated by cooking because it is heat stable, however, both the enzyme and the microorganisms can be inactivated (FDA, 2011).

Scrombroid fish are known to contain high levels of free histidine (Taylor *et al.*, 1989), which can be acted on by bacterial histidine decarboxylase. Examples of bacteria which can decarboxylate histidine include *Proteus morganii, Vibrio* and *Klebsiela pneumonia.* The growth temperatures of these microorganisms are between 8-15 ºC. Histamine formation is solely dependent on bacteria activity in fish and may serve as a proxy to microbial load. The quality and safety of fish is influenced by histamine production (Fletcher, 2010).

2.5 Bacteria responsible for histamine production in fish

There is a relationship between the formation of histamine and increasing bacterial load (WenXian *et al.*, 2001), however, merely a small percentage of the bacterial flora are histamine producing bacteria (example 12% in iced anchovies (Pons-Sanchez-Cascado *et al.*, 2005a)).

The bacteria responsible for histamine in fish have been identified as enteric and marine bacteria. However, the enteric *M. morganii* was identified to be the main contributor because it could produce high histamine levels (Shin-Hee *et al.*, 2003a) .In recent years, genotypic methods such as polymerase chain reaction (PCR) amplification have been used to identify bacterial isolates. These methods have been able to discover new histamine producing species.

Mesophiles, mostly, the *Enterobacteriaceae* family and a bit of *Pseudomonas* group were reported as main contributors of histamine production, until the year 2000. Recent work also support this finding showing that some decarboxylase positive bacterial species which produce histamine in test tuna fillet include; *Morganella morganii*, *Proteus vulgaris, Enterobacter intermedium, Pseudomonas fluorescen, Enterobacter agglomerans* and *Serratia liquefaciens* (Du *et al.*, 2002).

Sureelak *et al*, 2005, implicated *M. morganii*. In his work, he identified 28% of bacterial isolates from spoiled Indian anchovies as histamine producers. The histamine producers were; *Morganella morganii, Proteus vulgaris* and *Enterobacter aerogenes* (Sureelak *et al.*, 2005)

In a work done by Kim *et al*, 2002, they incubated some histamine producing microorganisms on fresh albacore tuna at 25 ºC for 3 days until counts reached 10 cfu/g. They found out that the weak histamine producers yielded histamine concentrations less than 350mg/kg. These weak histamine formers included *Hafnia alvei*, *Acinetobacter lwoffii, Photobacterium damsela*, *Enterobacter aerogenes* and *Enterobacter cloacae*. They also found that *M. morganii* could produce more than 3000 mg/kg of histamine, making it an important histamine producer (Kim *et al.*, 2002b). According to Kim *et al*, 2003, the main histamine producer in mackerel and sardines was *M. morganii* (Kim *et al.*, 2003). *M. morganii* has also been implicated in the production of other biogenic amine such as putrescine, cadaverine and phenylethylamine in tuna infusion broth.

Biogenic amines have been hypothesized to aid in histamine poisoning (Shin-Hee *et al.*, 2000).

2.6 Regulations on histamine levels in fish

The European Commission has set specifications on histamine in fish to regulate fish products in trade. It requires that nine samples of fish are analyzed and of these, not more than two should exceed 100 mg/kg and none should exceed 200 mg/kg (European Commission Regulation, 2005).

The draft Codex Alimentarius decomposition standard for smoked fish also requires that the histamine concentrations should not exceed 100 mg/kg, however, levels of 200 mg/kg may be used as critical limit when handling and hygiene are considered (Joint FAO/WHO Food Standards Programme Codex Alimentarius Commission, 2010). Both regulations are applied by the Ghana Standards Authority in its analysis of fish samples.

2.7 Scombroid poisoning

Histamine (or scombroid) poisoning is defined as an intoxication that results from the consumption of spoiled fish. Histamine poisoning is associated with fish species that have high levels of the amino acid; histidine. These types of fish are known to be in the Scombridae family and they include tuna, mackerel, sardine and bonito. The toxin associated with the poisoning is termed as scombrotoxin, although intoxication can also be caused by non-scomboid fish species. Histamine poisoning can cause allergic attacks which are short lived, however they are very unpleasant for affected persons (Fletcher, 2010).

Histamine poisoning will normally occur when fish having over 1000 mg/kg histamine is consumed, however, histamine levels as low as 200 mg/kg have been reported to cause the intoxication (Bartholomew *et al.*, 1987). Susceptible fish should be stored at temperatures below the minimum growth for mesophilic histamine producing bacteria or for periods which cannot allow psychrotrophic bacteria to reproduce and form unacceptable histamine levels (Fletcher, 2010).

2.7.1 Treatment

The best mode of therapy for histamine poisoning is antihistamine treatment which causes symptoms to subside quickly. H_1 antagonists such as diphenhydramine and H_2 antagonist such as cimetidine are examples of antihistamine treatment. In mild cases, pharmacological interventions are not necessary as the symptoms are short lived. Fluid replacement is one form of support for such patients (Taylor, 1986).

2.8 Toxicological aspects of histamine

2.8.1 Absorption, distribution, metabolism and excretion

Humans can ingest (up to) 180 mg of pure histamine without any obvious side effect, whereas, 0.007 mg of pure histamine administered intravenously could increase heart rate and cause vasodilation (FAO/WHO 2013).This variation proves that histamine is not efficiently absorbed from the gastrointestinal tract. It has been proposed that enzymes which metabolize histamine in the intestinal tract slow down the absorption of ingested histamine into the circulatory system (Taylor, 1986).

In humans, the enzyme histidine decarboxylase mediates histidine biosynthesis in mast cells, basophils, the gastric mucosa and histaminergic neurons. Endogenous histamine biosynthesis is strictly regulated; hence histidine decarboxylase is rapidly degraded when adequate histamine levels have been produced (FAO/WHO 2013).

In histamine producing cells, it is stored in a complex with heparin in secretory granules and released into circulation to exert their effect. Epidermal cells have recently been shown to also produce histamine in smaller quantities which are rapidly discharged into their surroundings (FAO/WHO, 2013)

Histamine metabolizing enzymes in humans include; diamine oxidase which converts histamine to imidazole acetic acid and is conjugated to ribose prior to excretion. On the other hand, histamineN-methyltransferase breaks down histamine into methylhistamine which is converted to Nimidazole acetic acid by monoamine oxidase (Maintz and Novak, 2007). Histamine metabolic byproducts are mainly excreted in the urine.

Diamine oxidase is a metabolic enzyme for ingested histamine because is it mainly found in the intestinal tract. Conversely, histamine-N-methyltransferase can only deal with histamine administered intravenously or intradermally. This is because the enzyme's main activity is concentrated in the liver (FAO/WHO, 2013)

Histamine metabolism can be altered when there is intake of isoniazid (FAO/WHO, 2013) and other drugs which slow down the action of Diamine oxidase and monoamine oxidase. Patients with tumours, mastocytosis, or chronic myelocytic leukaemia are also known to have altered histamine metabolism (Maintz and Novak, 2007). Histamine -N-methyltransferase is selective for histamine, whereas, other biogenic amines such as cadavarine and putrescine are also substrates for Diamine oxidase. Other impediments of histamine metabolism are ingested cadaverine, thiamine, and tyramine (Taylor, 1986).

It has been shown that about 68-80 percent of orally administered ^{14}C histamine is excreted through the urine while a proportion of the remaining is excreted in the faeces. The rest is then broken down by intestinal microbes and released as carbon dioxide through the lungs (Sjaastad and Sjaastad, 1974).

There are four different types of histamine receptors; (H_1, H_2, H_3 and H_4) in or on cell membranes of various cell types. Histamine binds to and activates these receptors eliciting multiple physiological responses such as vasodilatation and vasopermeability, flushing, hypotension and headache. In addition, histamine may also stimulate intestinal smooth muscle, resulting in abdominal cramps, diarrhoea and vomiting (Lehane and Olley, 2000).

2.8.2 Toxicological responses in humans

Endogenous histamine is essential for normal physiological functions, however in large doses, histamine becomes toxic affecting several organs (Taylor, 1986). The toxicological effects of histamine include:

1) Peripheral blood vessels which causes symptoms such as flushing, hypotension and headache. Increased vascular permeability associated with histamine intoxication also induces symptoms like haemoconcentration, urticaria and increased blood viscosity as well as shock. Histamine receptors H_1 and H_2 mediate increased vascular permeability (FAO/WHO, 2013). 2) Increased heart contractibility causing palpitation which is a hallmark in people experiencing histamine intoxication. Histamine may also cause contraction (mediated by H1 receptors) or relaxation (mediated by H2 receptors) of extravascular smooth muscle (Shahid *et al*, 2009).

3) Contraction of smooth muscle in the bronchi and the small intestine. In the intestines, this results in abdominal cramps, diarrhoea and vomiting often associated with histamine poisoning (Taylor, 1986).

4) Stimulation of sensory and motor neurons. This produces the symptoms such as pain and itching that accompanies urticarial lesions during histamine intoxication. The neurological effects of histamine is mediated by H_1 receptors (Nuutinen and Panula, 2010).

2.9 Effects of storage time and temperature on histamine levels in fish

Storage time and temperature are crucial to histamine formation in fish. Example; the shorter the time of storage, the higher the temperature required to attain a certain level of histamine concentration.

Research has also shown that to reach high and unacceptable levels of histamine concentrations, products have to be stored at high temperatures for long periods (Min-Ki *et al*. 2009).

In one experiment, tuna was stored at a temperature of 5 ºC for 8 days and reached a histamine concentration of only 6 mg/kg, conversely, mackerel and tuna which were stored at a temperature of 20 ºC and 30 ºC for 1 and 2 days respectively, attained histamine levels beyond 500 mg/kg (Ohashi, 2002). . In other reports, histamine concentrations of Atlantic mackerel stored at 25 ºC for 24 hours reached 100 mg/kg whereas, those held at 4 ºC for 3 days did not attain such high concentrations (Merialdi *et al.*, 2001).

Histamine concentrations in fish can decrease during storage at ambient temperatures for relatively long periods of time. This is possible through the action of bacteria which have histaminases activity that dominate only when the fish is spoilt (Taylor, 1986).

2.10 Effect of histamine on sensory characteristics of fish

According to Antoine *et al*, (2004), there is a relationship between sensory deterioration and histamine levels. Concentrations of histamine and other biogenic amines are used as quality indicators and as they increase, sensory quality also decreases (Antoine *et al.*, 2004, Jeya Shakila *et al.*, 2001).

Sensory panelist rejected yellowfin tuna (*Thunnus albacares*) before it reached unacceptable histamine levels (Guizani *et al.*, 2005). They found out that histamine concentrations reached unacceptable levels after 1 day of storage at 20 ºC or 4 days at 8 ºC. Another work done on whole

ungutted sardine (*Sardina pilchardus*) by Erkan and Ozden, (2008), showed that the sensory panelist rejected the fish after 7 days of storage in ice. The histamine concentration exceeded critical limit (Erkan and Ozden, 2008).

Notwithstanding, the sensory evaluation is not always dependable in assuring safety of fish (Fletcher *et al.*, 1995). To support this assertion, tuna stored for days and attained histamine levels of about 1000 mg/kg were considered acceptable from a sensory perspective (Du *et al.*, 2002). The odour of bluefish (*Pomatomus salatrix)* were accepted even though some were inoculated with *M. morganii* (Lorca *et al.*, 2001).

2.11 Control of histamine formation

2.11.1 Refrigeration

A recommended technique used in preventing histamine contamination is rapidly chilling fish that are prone to slow down or inhibit the growth of these histamine producing bacteria. The US Sea Extension program applies the following recommendations to ensure integrity of fish.

- Harvested Fish should be immediately stored in ice or refrigerated sea water or brine at 4°C or less within 12 hours of death or 10 °C or less within 9 hours of death.
- Large fish (i.e. above 9 kg) that are eviscerated before chilling on board should be kept in ice or refrigerated sea water or brine.
- Large Tuna (i.e. above 9 kg) which are not eviscerated before chilling on board should be chilled to an internal temperature of 10°C or less within 6 h of death (Lampila and Tom, 2009).

2.11.2 Heading, gutting and skinning

Histamine producing bacteria are normally found in the guts, gills and skin of freshly harvested fish. It is therefore necessary to remove any of these parts of fish to delay histamine production (Pons-Sanchez-Cascado *et al.*, 2003).

2.11.3 Heat processing

Processes which require heat treatment such as canning cannot affect histamine concentration. This implies histamine is heat stable (Erkan *et al.*, 2001, Hyoungill *et al.*, 2005). However, in 2001, there was an outbreak of histamine poisoning in Taiwan and canned mackerel was implicated. The canned fish contained 1540 mg/kg histamine (Yung-Hsiang *et al.*, 2005b). Histamine forming bacteria are not detected in canned products and as such scombroitoxin identified in canned product are most probably produced before canning.(Yung-Hsiang *et al.*, 2005a)

Some research work also concluded that even though histamine is not eliminated, it is reduced by canning (Baygar and Gokoglu, 2004), hence, heat processing lessens the risk to some degree.

Some heat processing which does not meet canning requirements can be used as a method for deactivating histamine producing bacteria before formation of histamine (Emborg and Dalgaard, 2008),

2.11.4 Modified atmosphere packaging

Research has shown that some modified atmosphere packaging (MAP) can decrease the formation of histamine in susceptible products. A mixture of CO_2:O_2:N_2 (40:40:20) was found to prevent histamine production in big eye tuna (*Thunnus obesus*) whiles another composite; 60:15:25, resulted in a high histamine concentration exceeding 100 mg/kg, during a 33 days' storage at 2°C (Ruiz-Capillas and Moral, 2005). Mohan *et al,* 2009, found that oxygen scavenger which was used to remove oxygen from packs, significantly reduced histamine formation in seer fish whiles

enhancing its shelf life (Mohan *et al.*, 2009). Organic acids can be used to decrease pH and salt or other means can also be used to reduce water activity. These conditions and storage under high carbon dioxide environment can inhibit growth of histamine producing bacteria (Emborg and Dalgaard, 2008).

2.11.5 Freezing and chilled storage

Histamine formation in fish can be decreased by freezing. Freezing prevent the growth of histamine forming bacteria and reduces the activities of preformed histidine decarboxylase (Rossano *et al.*, 2006). According to (Staruszkiewicz *et al.*, 2004) the activity of the enzyme histidine decarboxylase could be maintained in frozen fish and could increase histamine concentrations upon thawing. Another study by (Yung-Hsiang *et al.*, 2005c) indicated that freezing halted histamine production and formation was resumed when fish was thawed.

Histamine could be accumulated rapidly exceeding 500 mg/kg within 36 h at 25 °C hence freezing was a hindrance to histamine production while product was frozen but will not necessarily prevent its occurrence in thawed fish.

2.11.6 Gamma irradiation

Irradiation can hinder production of histamine producing bacteria such as *M. morganii* in mackerel fillets (Aytac *et al.*, 2000). Other studies also showed that it reduced histamine content in bonito in a dose dependent fashion (Mbarki *et al.*, 2008). Atlantic horse mackerel (*Trachurus trachurus*) which was stored in ice for 23 days had its histamine concentration approaching 100 mg/kg whiles fish which had been irradiated at 1 kGy within the same time had no histamine (Mendes *et al.*, 2005). This confirms that irradiation is an option to control histamine contamination in fish.

2.11.7 Protective cultures of bacteria

While histamine is produced by bacteria that produce histidine decarboxylase, other bacteria produce diamine oxidases which can degrade histamine. Two such lactic acid bacteria were proposed by Enes Dapkevicus *et al.*, 2000 to be used as starter culture for fish silage production. Similar bacteria could be used in other fermented sea food products to control the production of histamine in seafood. *Staphylococcus xylosus* was proposed as a protective organism that could be used as a starter culture to prevent histamine production in Korean fermented foods as well as other products (Jae-Hyung *et al.*, 2008).

2.12 Fish consumption patterns

Fish consumption patterns are comparatively higher in African countries along the coastal belt than those in the land lock countries. In West Africa, countries along the coastal region have an annual fish consumption rate of about 20 kg per capita. Table 2 shows the fish consumption patterns of some selected African countries including Ghana.

Table 2. Fish consumption patterns

Country	Fresh/Frozen %	Smoked %	Fried %	Fermented		Per Capita Consumption kg/annum
				Salted %	Unsalted %	
Burundi	25	5	negligible	negligible	70	6.0
Chad	10	45	negligible	0	45	8.5
Côte d'Ivoire	35	50	5	10	0	17.7
The Gambia	30	55	5	10	0	16.4
Ghana	20	60	5	10	5	20.0
Mali	10	60	negligible	0	30	7.1
Senegal	70	20	negligible	10	0	20.7
The Sudan	70	5	negligible	10	15	2.0
Uganda	45	40	2	3	10	13.0

Source: Essuman, 1992

According to Essuman, (1992), about 80 percent the total fish supply in Ghana is cured using a variety of methods before they are consumed. Smoked fish is a very common cured fish widely used in indigenous stews and soups in the country. Smoking is also the main method used for fish preservation and it is preferred because of the flavor it imparts in fish. Other reasons for preference of smoked fish are its availability and possibility of storing for a few days. Most Ghanaians prefer their fish intact in soups and the smoked fish is able to serve that purpose unlike the fresh fish. Other processes used in curing fish are; sun drying, salting, partially fermenting and frying (Essuman, 1992).

2.13 Selected species of scombroid fish

2.13.1 The Atlantic Mackerel (*Scomber scombrus)*

The Atlantic mackerel (*Scomber scombrus*) also known as Boston mackerel or just Mackerel, is a pelagic schooling species which can be found on both sides of the North Atlantic Ocean. Both the male and female Atlantic mackerel grow about the same rate having a maximum fork length of about 47 centimeters (19 inches). The species are sexually matured by three years and have life span of about 20 years. The teeth are arranged in two rows and are widely spaced. The second dorsal fin is greater (approximately 1.5 times) than the length of the groove between itself and the second dorsal fin. The anal fin originates near the second dorsal fin and has a conspicuous spine while the swimbladder is absent. There are oblique to near vertical markings on the back while the belly lacks any markings (Collette and Nauen, 1983).

Figure 2. The Atlantic Mackerel (http://www.fisheries.no/ecosystems-and-stocks)

2.13.2 Atlantic Horse Mackerel (*Trachurus trachurus)*

This fish is a jack mackerel species and in the Carangidae family. It is an important commercial edible mackerel which can be smoked, fried, salted and grilled. It has an elongated body which is slightly compressed. It has a large head with its lower jaw projected and posterior end of its jaw extending to the anterior margin of the eye. The nostrils are small and close to each other. It has gillrakers which constitutes rudiments on the lower limb. The pelvic fin is moderate in size and originates below end of the pectoral fin base. Its scales are curved in lateral lines and are expanded dorsolaterally. It has no distinctive colour except for a small, black opercular spot on edge near upper angle. The Upper part of body and top of its head are black or grey to bluish green. About 60-70 percent of its body is generally pale and whitish or silvery (http://www.fao.org)

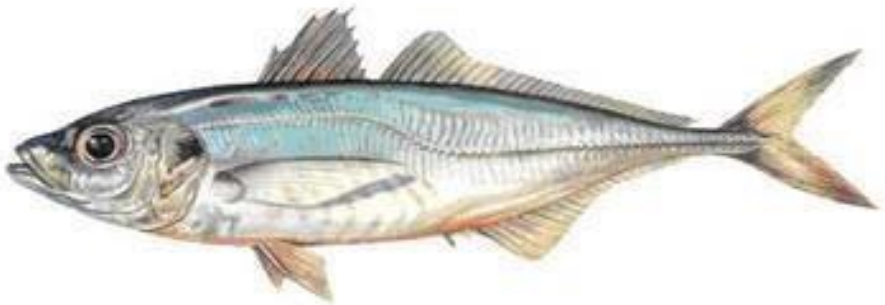


Figure 3. The Atlantic Horse Mackerel (http://www.baltlanta.lt)

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Study area

The study was carried out in Nungua, a coastal town in the Ledzokuku-Krowor Municipal district in the Greater Accra Region. Nungua is located at the south eastern part of Ghana near the coast. It is the eighteenth most populous settlement in Ghana, with a population of over 84,000 people (https://en.wikipedia.org).

Figure 4. A map of Accra showing the location of Nungua

Since it is a coastal town, inhabitants are noted to have fish as their main source of protein. The town is also known for Ga *kenkey* which is normally accompanied with grilled Atlantic horse mackerel (*shito loo*) and other kinds of fried fish.

A total of eight (8) coldstores and five (5) smoked fish sellers were randomly selected for the study. Most of the coldstores were concentrated in the Nungua market and had no electricity power backup. At the time of sampling, there was power outage; however the fish were still frozen. Interaction with the cold store attendants showed that their freezers could keep their fish frozen for the period of power outage which often lasted for a maximum of 24 hours.

3.2 Materials

The following materials were required for the analysis of histamine in fish samples; Portlab blender, Top load balance (precision 0.001 g), Centrifuge, Homogenizer ULTRA TURRAX , Syringe filter 0.45 µm, Centrifuge tubes, UFLC Shimadzu HPLC, Column TECNOKROMA C18 5 µm 250 x 4.6 mm, 1 coarse crushing equipment, blenders, fluorescence detector, pump for postcolumn reaction, membrane filter, pore size 0.45 microns disposable syringe delivery of 10 ml HPLC Vial , Histamine Dichloride RPE purity 98 %, RS Acetonitrile for HPLC, Perchloric acid 70% RE, 1-Octanesulfonic acid sodium salt, Sodium acetate, Sodium acetate trihydrate, Acetic acid, Boric acid, Potassium hydroxide, Polyoyethylene lauryl ether (Brij 35), Ortho – phthaldialdehye (OPA), 2 – Mercaptoethanol and Deionised water.

Other reagents include;

Eluent A:

8.03 g sodium acetate was dissolved in 800 mL deionised water in a beaker and the pH of the solution adjusted to 4.5 ± 0.1 with acetic acid. To this was added 2.16 g of sodium octane and the solution was made up with deionised water in a volumetric flask to 1000 mL.

Eluent B:

12.73 g sodium acetate was dissolved in 600 mL water (beaker) and the pH of the solution was adjusted with acetic acid to pH 4.5 ± 0.1. To this was added 2.16 g of sodium octane and 230 mL of acetonitrile (measuring cylinder) and the solution made up with deionised water in a volumetric flask to 1000 mL.

Borate buffer:

61.8 g of boric acid was dissolved with 40 g of potassium hydroxide in water in a volumetric flask and made up to 1000 mL mark with deionised water.

Derivatization

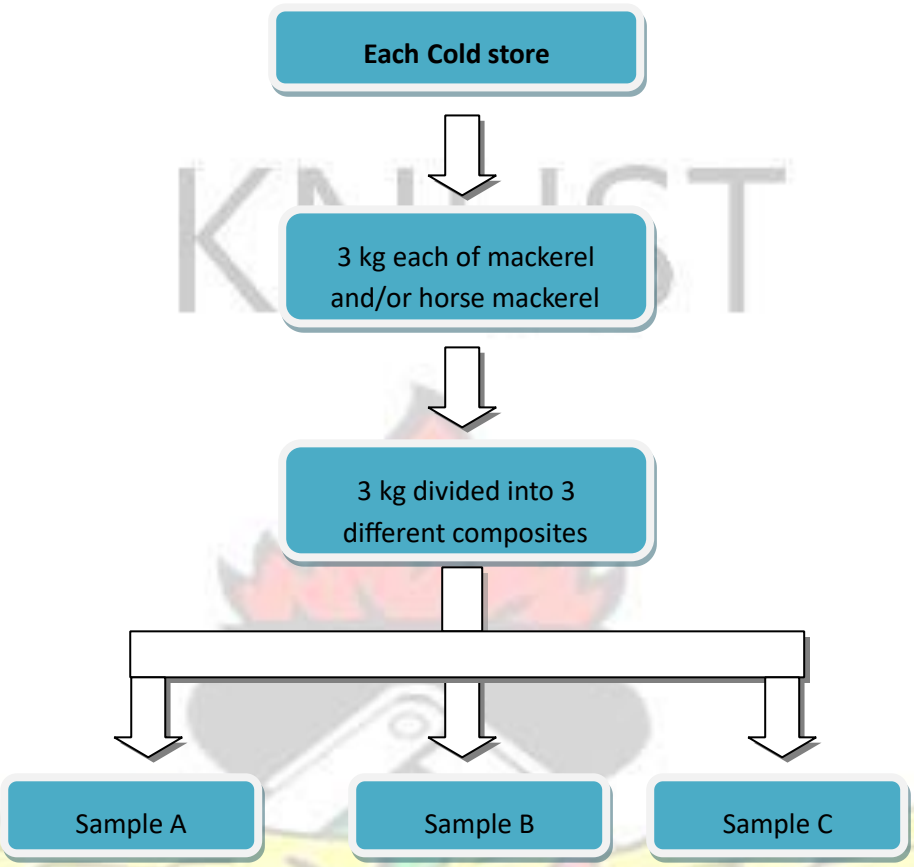
3 g of Brij and 1 g of o-phthaldialdehyde were weighed into a small beaker and dissolved in 10 mL of methanol. The resulting solution was added to 1 L of borate buffer (graduated cylinder). After addition of 3 mL of 2-mercaptoethanol, the solution was ready for use in an amber flask.

3.3 Methods

3.3.1 Experimental design, sampling and data collection

Two (2) species of fish namely; Atlantic horse mackerel (locally known as *Kpanla*) and Mackerel (Ghanaian Salmon) were purchased from cold stores in the Nungua on 19th October, 2015. Smoked fish samples were also purchased from the five (5) different sellers on 6th of February, 2016.

The



species selected for the analysis were based on their popularity among local consumers and availability at the time of sampling. The frozen fish samples were transported to the laboratory in ice chest with ice packs and stored in the freezer until they were analyzed. However, the smoked fish were transported in an ambient temperature and analyzed immediately upon receipt into the laboratory

Three (3) different composite of each species of fish from each coldstore were analyzed for histamine. The frozen fish were cleaned; deboned and eviscerated whiles the smoked fish were scaled and deboned. A high speed blender was used to obtain a homogenous sample.

A total of eighteen blended frozen fish samples and five smoked fish samples were prepared for histamine analysis.

Figure 5. A flow chart for sampling for frozen fish in the Nungua coldstores for histamine analysis

Altogether, fish was sampled from eight (8) different coldstores.

3.4 Procedure for histamine Analysis

3.4.1 Overview

The method involved extraction of histamine from fish in perchloric acid solution. The sample was homogenized with Ultra Turrax homogenizer, followed by centrifugation and filtration. The concentration of histamine was determined by high performance chromatographic (HPLC) method with ultra violet (UV) detector.

3.4.2 Extraction

Extraction was done according to the Histamine Laboratory In-house method, Ghana Standards Authority, *unpublished*. Each test was performed by weighing 10 g blended sample in a 100 mL beaker. Extraction solution (20 mL) was added and homogenized with Ultra Turrax for 2 minutes. The sample was transferred into a 50 mL volumetric cylinder and filled up to the mark with 0.6 perchloric acid using Pasteur pipette to wash beaker and Ultra Turrax. The extracts were filtered with a 541 filter paper. The filtrates were then transferred again into tubes and centrifuged for 5 minutes at 3000 rpm. The supernatant solution was filtered into vials with of
0.45 microns syringe filter.

3.4.3 Preparation of standard solutions

3.4.3.1 Preparation of stock solution of histamine (histamine 1000 mg/kg)

Histamine dichloride (0.166 g) was weighed and transferred to a 100 ml volumetric flask. The volume was made up to the 100 mL mark with deionized water. The standard solution was refrigerated afterwards until use.

3.4.3.2 Preparation of working standard

Histamine 100 mg/kg: 10 mL of the 1000 mg/kg solution was pipetted into 100 mL volumetric flask and topped to the mark with deionized water.

3.4.3.3 Preparation of calibration standard solution

Table 3. Standard solution preparation

Initial concentration (mg/kg)	Aliquot (ml)	Final volumetric flask (mL)	Final concentration (mg/kg)
1000	0.2	100	2
1000	0.5	100	5
1000	1	100	10
1000	2	100	20
1000	3	100	30

Volumes of working standard (aliquots) required to prepare the various concentrations of the calibration curve standard solution are indicated.

3.4.4 HPLC analysis

- **Chromatographic conditions**

Injection volume: 50 µl

Eeluent A and eluent B: 0.7 mL/min

Derivatization reagent: 0.5 mL/min

Elution: isocratic, Phase A: Phase B, 15:85

Fluorescence detection: excitation 330 nm, emission 465 nm Rinse
Autoinject: Methanol 50%, water 50%

- **Preparation of calibration curve**

A calibration curve was prepared by injecting standard solutions.

Calculation

The concentration of histamine in fish products was expressed as mg/kg (ppm). The histamine peak was identified by comparing the retention times of standard solutions. The value obtained was multiplied by 5 (extraction factor-EF) to obtain the result in mg/kg (ppm) (Histamine Laboratory In-house method, Ghana Standards Authority, *unpublished*).

3.5 Statistical analysis

The data obtained were analyzed with Microsoft Office Excel 2007. Levels of histamine concentrations within each fish species from the different cold stores were compared using one-way ANOVA.

CHAPTER FOUR

4 RESULTS AND DISCUSSION

4.1 Study samples

A total of 3 kg each of frozen Atlantic mackerel and frozen Atlantic horse mackerel were purchased from 8 different coldstores respectively and analyzed. Four (4) of the coldstores, designated; Seller 1, Seller 2, Seller 3 and Seller 4 respectively had in stock both fish species. Of the remaining coldstores, 2 (Seller 5a and Seller 6a) had only Atlantic mackerel in stock while the other 2 (Seller 5b and Seller 6b) had only Atlantic horse mackerel in stock. In addition, five smoked samples of each species were also analyzed. At the time of sampling for the frozen fish, there was power

outage; however, upon interactions with the coldstore operators, it was revealed that their freezers could keep the fish frozen for at least 12 hours in the absence of electrical power. Thus all the fish bought from the coldstores were in their frozen state with no signs of thawing.

4.2 Histamine concentrations in frozen Atlantic Mackerel

The histamine concentrations of the frozen Atlantic mackerel are shown in figure 6. Atlantic mackerel from 4 out of the 6 coldstores recorded no histamine concentrations. The concentrations ranged from 0 - 3 mg/kg. There was no significant difference (p>0.05) in histamine levels found in fish obtained from the different sellers. Histamine production is suspended at frozen temperatures (-18 ºC or below) (FDA, 2011). The absence or low concentration of histamine may be attributed to the inactivation of the enzyme decarboxylase and histamine producing bacteria due to adequate storage.

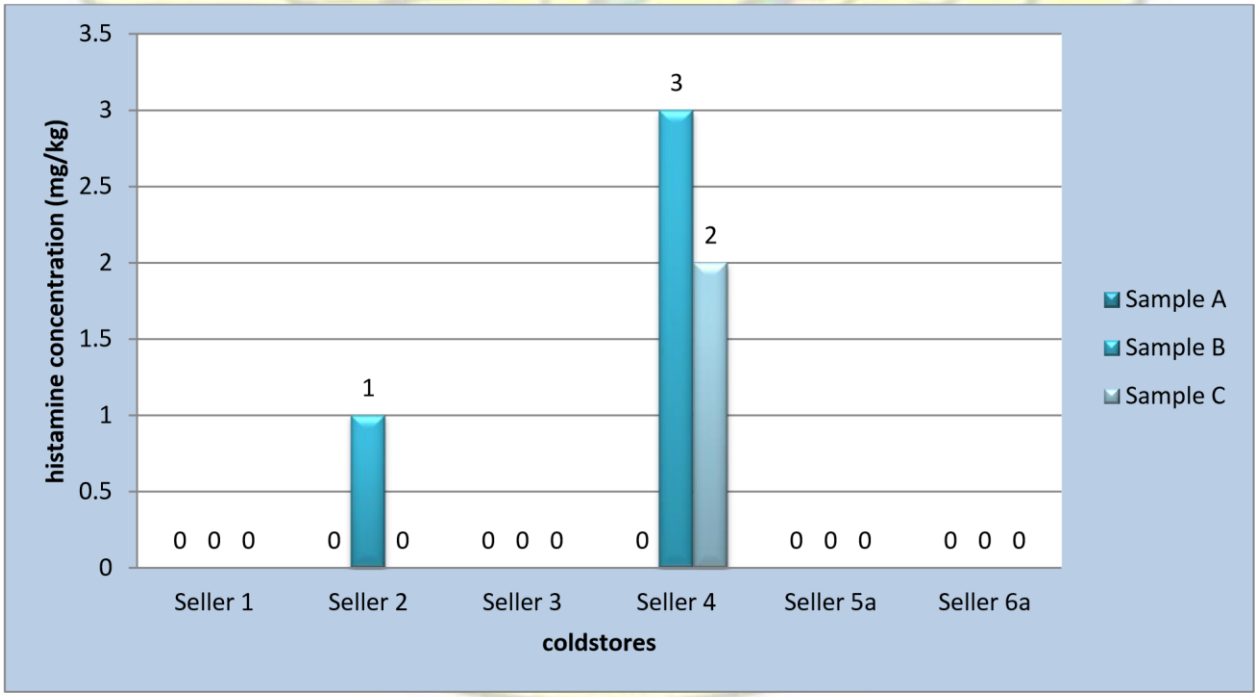


Figure 6. Histamine concentrations of frozen Atlantic Mackerel from cold stores in Nungua market

4.3 Histamine concentrations in frozen Atlantic Horse Mackerel

The histamine concentrations of the frozen Atlantic horse mackerel are shown in figure 7. Atlantic horse mackerel from all the 6 coldstores except 1 recorded some histamine levels. Histamine concentrations of the Atlantic Horse Mackerel could be considered as low because they did not exceed the critical limit of 100 mg/kg (Commission of the European Communities, 2005). The range of means for the 6 sellers was 0 - 20.67 mg/kg. There was a significant difference (p<0.05) in the histamine levels in fish from the different sellers, suggesting that different cold storage conditions may pertain in the different coldstores. Seller 6b in particular recorded higher levels of histamine compared to the other sellers. Although, all samples appeared well frozen at the time of purchase, it is not certain if the cold chain was equally maintained in all coldstores. The current study did not assess microbial levels in the fish samples, however the different levels of histamine measured indicated that some histamine producing bacteria may be present in some of the fish samples. Histamine can be produced at or near refrigeration temperature so far as the histidine decarboxylase has been formed (FDA, 2011). Therefore, if the fish handling processes prior to freezing were not appropriate, the preformed histidine decarboxylase will mediate histamine formation in sub-optimal freezing conditions.

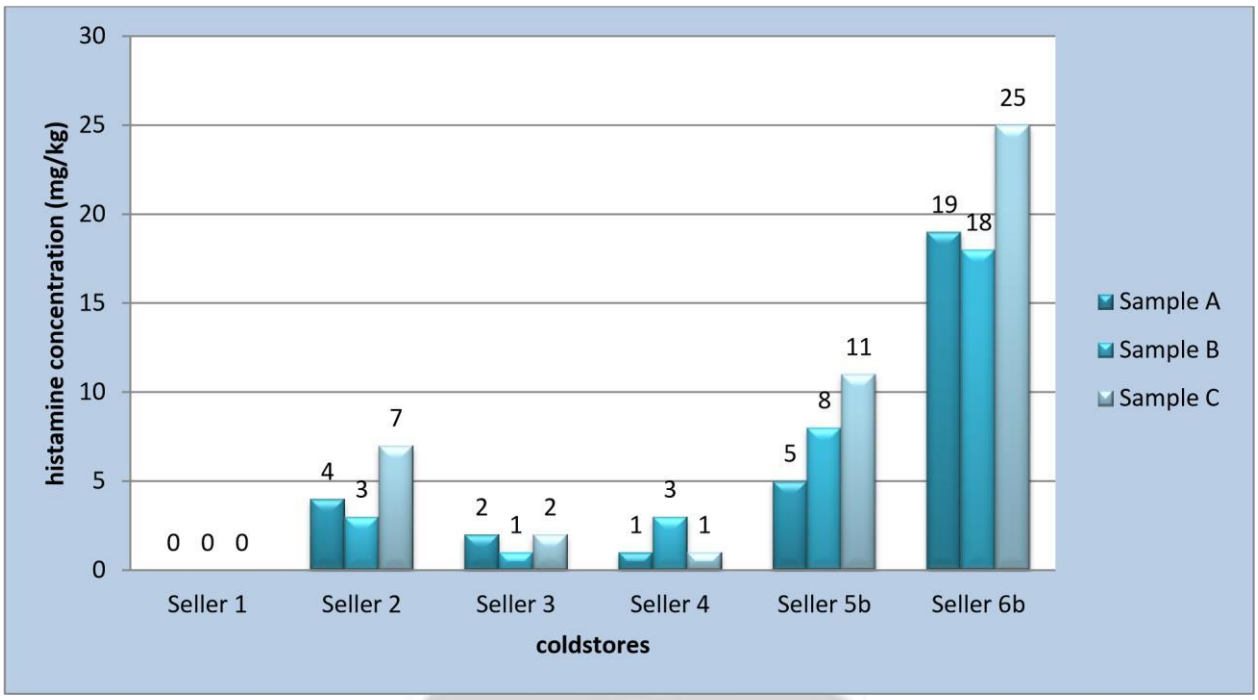


Figure 7. Histamine concentrations of frozen Atlantic Horse Mackerel from cold stores in Nungua market

4.3 Histamine concentrations in smoked Atlantic Mackerel

Figure 8. Histamine concentrations of smoked Atlantic Mackerel from Nungua market

The graph (figure 8) shows the histamine concentrations obtained from smoked Atlantic mackerel. The lowest concentration recorded was 13 mg/kg and the highest was 27 mg/kg. These concentrations, even though do not exceed the critical limit of 200 mg/kg (Joint FAO/WHO Food Standards Programme Codex Alimentarius Commission, 2010) confirms the presence and activities of histamine producing bacteria before the fish was smoked.

4.4 Histamine concentrations in smoked Atlantic Horse Mackerel

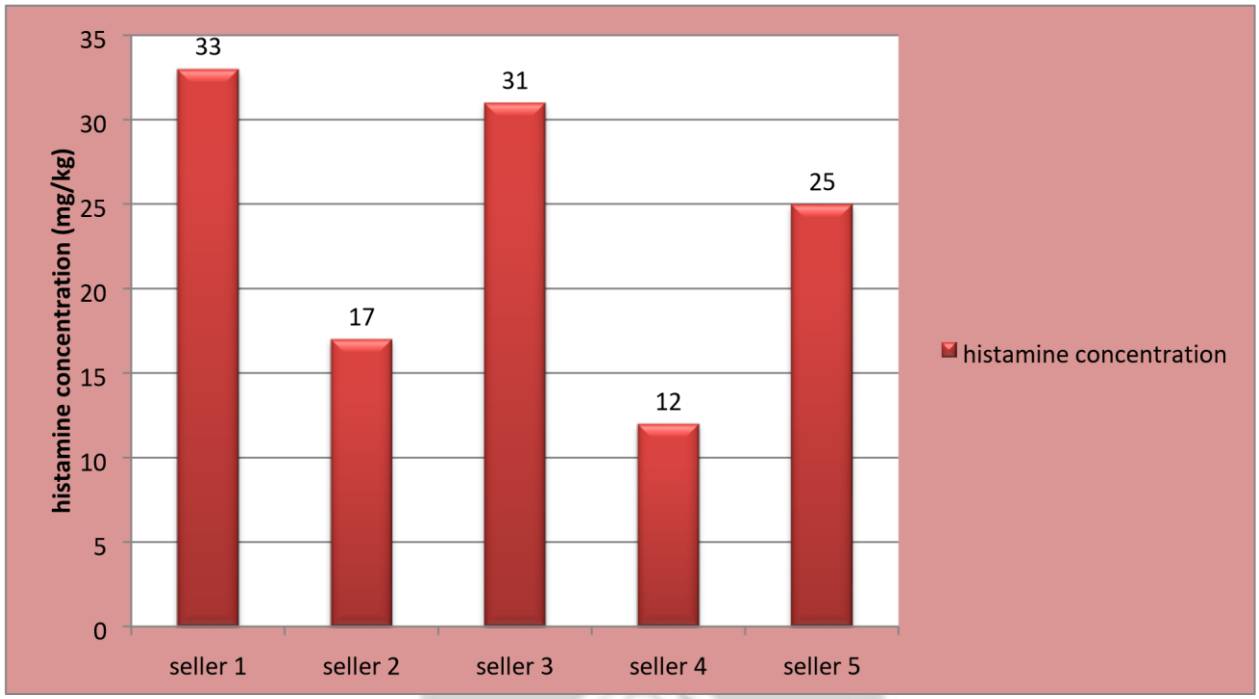


Figure 9. Histamine concentrations of smoked Atlantic Horse Mackerel from Nungua market

The histamine concentrations of smoked Atlantic horse mackerel are shown in figure 9. The lowest concentration was 12 mg/kg and the highest was 33 mg/kg. Fish are usually smoked almost immediately after purchase from fishermen/from coldstores. In this case, the species of fish involved are usually imported therefore; they are received in the frozen state. Frozen fish are thawed before they are smoked; hence, formation of histamine may depend on the time used in thawing the fish. The smoking stops the activities of histamine forming bacteria as they are destroyed by heat, however, any preformed histamine cannot be destroyed at this stage.

Informal interactions between the sellers of smoked fish /fish smokers and this researcher revealed that they had knowledge of the implications of inadequate temperature storage; however, they did not know the mechanisms of spoilage. The basic knowledge they had aided them in handling fish very well before they were smoked.

The levels of histamine obtained from the analysis of both frozen and smoked fish may imply that those species of fish in the Nungua markets are safe for consumption (in relation to histamine contamination).

CHAPTER FIVE

5 CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

Both frozen Atlantic mackerel and Atlantic horse mackerel had low levels of histamine. However, the smoked Atlantic mackerel and Atlantic horse mackerel had higher levels of histamine in them but this was not based on any formal statistical comparison due to the low sample size. All concentrations determined were within acceptable limit implying the selected species of fish were safe for consumption in relation to histamine concentrations.

5.2 RECOMMENDATIONS

1. Research on histamine levels in fish should be extended to other species of scombroid fish such as sardinella, tuna, etc. in other parts of the country. If concentrations of histamine obtained in the studies are significant or exceed critical limits, exposure assessment can be carried out.
2. Future studies should involve larger sample size and more study areas
3. It is evident that safe practices are being employed in the fish market and so as a reminder the following should be done to maintain the low histamine levels in fish.
    a. The cold chain storage of fish should be maintained from point of receipt/port to their destinations/coldstores.

LIMITATIONS

The method used for histamine analysis required some expensive chemicals making the cost of analysis expensive. This limited the amount of samples which could be used in the project.

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APPENDICES

Appendix I (Histamine concentrations of fish)

Histamine concentrations of frozen mackerel

	Sample A (mg/kg)	**Sample B (mg/kg)**	**Sample C(mg/kg)**
Seller 1	Not detected	Not detected	Not detected
Seller 2	Not detected	1	Not detected
Seller 3	Not detected	Not detected	Not detected
Seller 4	Not detected	3	2
Seller 5	Not detected	Not detected	Not detected
Seller 6	Not detected	Not detected	Not detected

Histamine concentrations of frozen horse mackerel

	Sample A (mg/kg)	**Sample B (mg/kg)**	**Sample C (mg/kg)**
Seller 1	Not detected	Not detected	Not detected
Seller 2	4	3	7
Seller 3	2	1	2

Seller 4	1	3	1
Seller 5	5	8	11
Seller 6	19	18	25

Histamine concentrations of smoked mackerel

	Histamine concentration (mg/kg)
Seller 1	18
Seller 2	13
Seller 3	24
Seller 4	27
Seller 5	15

Histamine concentrations of smoked horse mackerel

	Histamine concentration (mg/kg)
Seller 1	33
Seller 2	17
Seller 3	31
Seller 4	12
Seller 5	25

Appendix Two (Results of Data Analysis using One-Way Anova)

Frozen mackerel

SUMMARY	*Count*	*Sum*	*Average*	*Variance*
Seller 1	3	0	0	0
Seller 2	3	1	0.333333	0.333333
Seller 3	3	0	0	0
Seller 4	3	5	1.666667	2.333333
Seller 5	3	0	0	0
Seller 6	3	0	0	0

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Sample A	6	0	0	0
Sample B	6	4	0.666667	1.466667
Sample C	6	2	0.333333	0.666667

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1.333333	2	0.666667	0.9375	0.413386	3.68232
Within Groups	10.66667	15	0.711111			
Total	12	17				

Frozen Atlantic horse mackerel

Anova: Single-Factor

SUMMARY	Count	Sum	Average	Variance
Seller 1	3	0	0	0
Seller 2	3	14	4.666667	4.333333
Seller 3	3	5	1.666667	0.333333
Seller 4	3	5	1.666667	1.333333
Seller 5	3	24	8	9
Seller 6	3	62	20.66667	14.33333

Anova: Single Factor

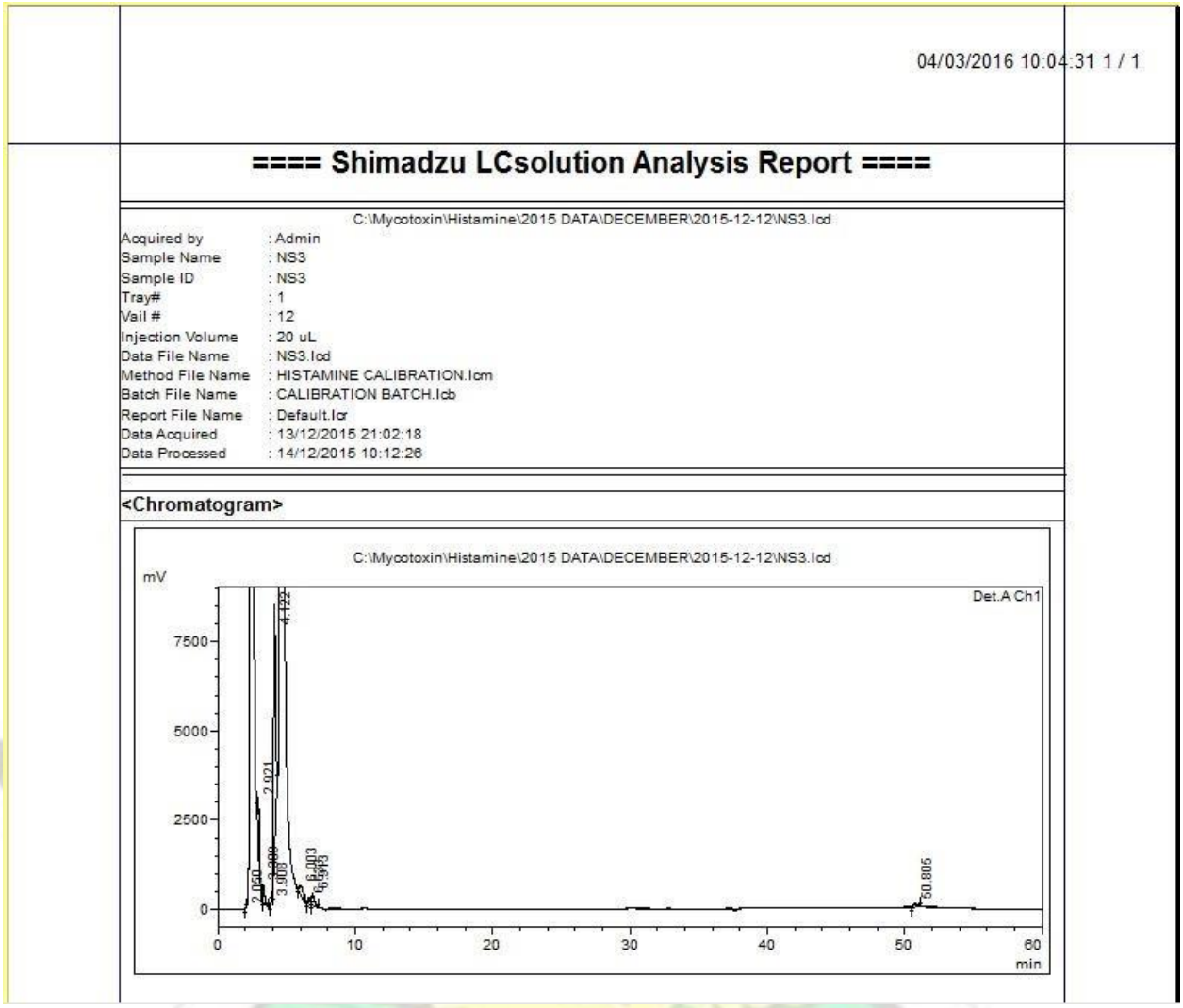
SUMMARY

Groups	Count	Sum	Average	Variance
Sample A	6	31	5.166667	49.36667
Sample B	6	33	5.5	45.1
Sample C	6	46	7.666667	89.46667

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	22.11111	2	11.05556	0.180319	0.836787	3.68232
Within Groups	919.6667	15	61.31111			
Total	941.7778	17				

Appendix Three –A chromatogram showing the histamine peak of frozen Atlantic mackerel



A chromatogram showing the histamine peak of frozen Atlantic horse mackerel

04/03/2016 09:59:09 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Mycotoxin\Histamine\2015 DATA\DECEMBER\2015-12-12\NK2.lcd

Acquired by	: Admin
Sample Name	: NK2
Sample ID	: NK2
Tray#	: 1
Vail #	: 15
Injection Volume	: 20 uL
Data File Name	: NK2.lcd
Method File Name	: HISTAMINE CALIBRATION.lcm
Batch File Name	: CALIBRATION BATCH.lcb
Report File Name	: Default.lcr
Data Acquired	: 14/12/2015 00:03:42
Data Processed	: 14/12/2015 10:12:23

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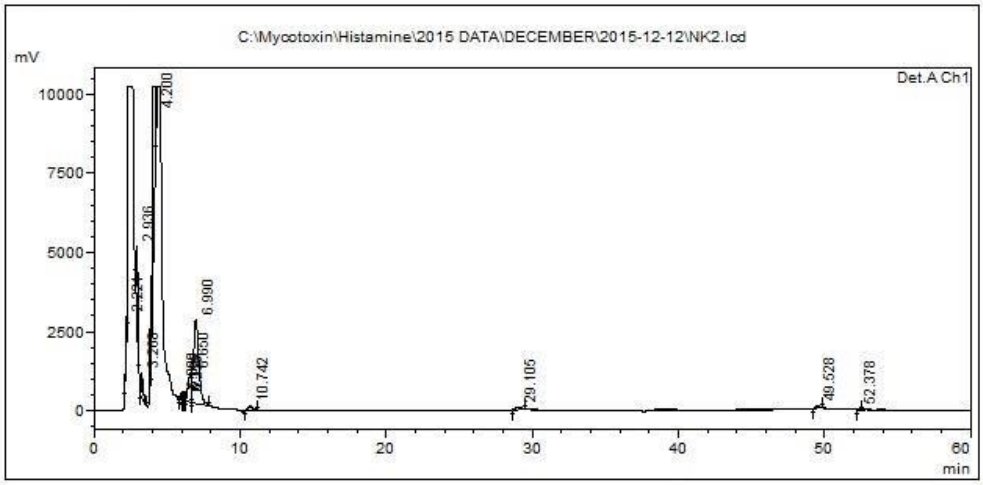

A chromatogram showing the histamine peak of smoked Atlantic mackerel

04/03/2016 10:06:51 1 / 1

==== Shimadzu LCsolution Analysis Report ====

C:\Mycotoxin\Histamine\2016 DATA\FEBRUARY\2016-02-14\A1.lcd

Acquired by	: Admin
Sample Name	: Smoked salmon
Sample ID	: A1
Tray#	: 1
Vail #	: 78
Injection Volume	: 20 uL
Data File Name	: A1.lcd
Method File Name	: HISTAMINE CAL METHOD.lcm
Batch File Name	: CAL BATCH.lcb
Report File Name	: Default.lcr
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Data Processed	: 14/02/2016 14:29:12

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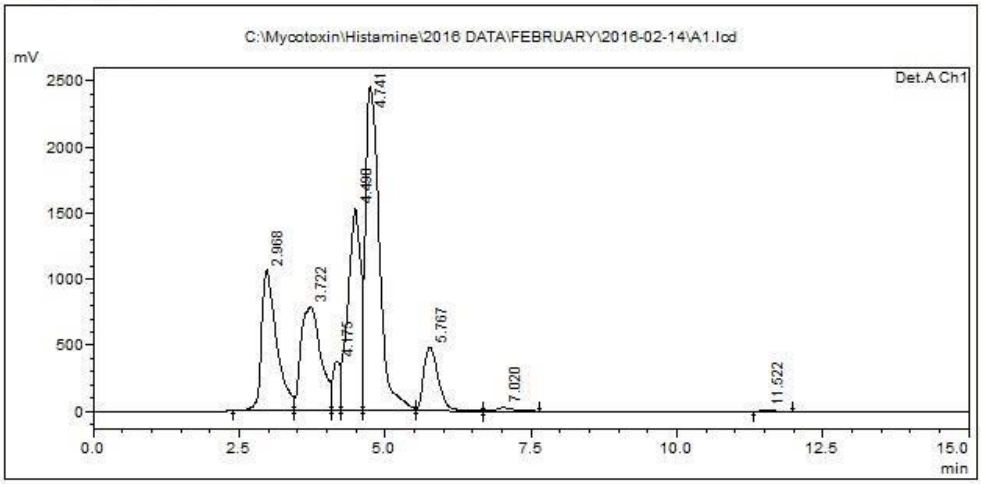

A chromatogram showing the histamine peak of smoked Atlantic horse mackerel

04/03/2016 10:08:04 1 / 1

==== Shimadzu LCsolution Analysis Report ====

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Acquired by	: Admin
Sample Name	: Smoked Kpanla
Sample ID	: A4
Tray#	: 1
Vail #	: 81
Injection Volume	: 20 uL
Data File Name	: A4.lcd
Method File Name	: HISTAMINE CAL METHOD.lcm
Batch File Name	: CAL BATCH.lcb
Report File Name	: Default.lcr
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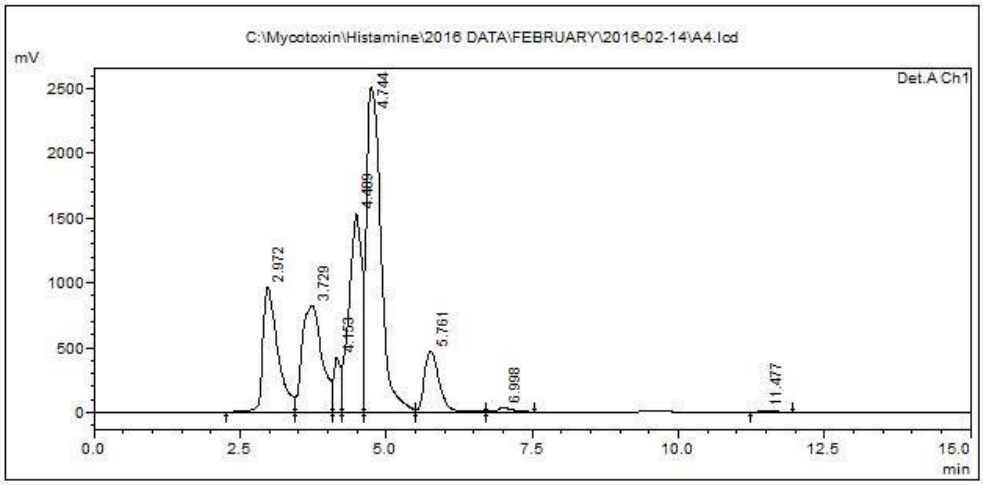

A chromatogram showing the histamine peak of Certified Reference Material

==== Shimadzu LCsolution Analysis Report ====

C:\Mycotoxin\Histamine\2016 DATA\FEBRUARY\2016-02-14\27132.lcd

Acquired by	: Admin
Sample Name	: CRM
Sample ID	: 27132
Tray#	: 1
Vail #	: 76
Injection Volume	: 20 uL
Data File Name	: 27132.lcd
Method File Name	: HISTAMINE CAL METHOD.lcm
Batch File Name	: CAL BATCH.lcb
Report File Name	: Default.lcr
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