EVALUATION OF BIOGENIC AMINE AS A QUALITY INDEX IN INDIAN SQUID (*Loligo duvauceli*)

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By

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This is to certify that this thesis entitled "**Evaluation of Biogenic Amine as a quality index in Indian Squid (*Loligo duvauceli*)**" embodies the original work conducted by Mrs.Anju K.A under my guidance. I further certify that no part of this thesis has previously been formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or in any other university or Institution. She has also passed the Ph.D qualifying examination of the Cochin University of Science and Technology, Cochin held in January 2010.

Date:

Dr. T.V.Sankar,
(Supervising Guide)

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Declaration

I, Anju K.A. do hereby declare that the thesis entitled "**Evaluation of Biogenic Amine as a quality index in Indian Squid (*Loligo duvauceli*)**" is a genuine record of bonafide research carried out by me under the supervision of Dr. T.V.Sankar, (Principal Scientist, ICAR-CIFT) & Director of Research, Kerala University of Fisheries and Ocean studies, Cochin and has not previously formed the basis of award of any degree, diploma, associateship, fellowship or any other similar titles of this or any other university orInstitution.

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GENERAL INTRODUCTION

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1.1 Introduction

Fish harvesting and fish processing in India is a major industry, providing nutritional security to the food basket, contributing to the agricultural exports and engaging about fourteen million people in different activities. Fish production in India has increased more than tenfold since its independence in 1947. Seafood is one of major source of foreign exchange earnings.

1.2 Fisheries Production

Accounting for about 5.4% of the worldwide fish production, India today is the second leading fish producing nation in the world (FAO, 2017). India is also a major producer of fish through inland and ranks second in global fish production after China. According to the estimates of the year 2015-16 there was about Rs. 1 lakh crore value fish production in the country. The figure for total fish production in India during 2014-15 is 10.06 Million Tonnes (MT) with a contribution of 6.57 MT from aquaculture sector and 3.49 MT from Marine sector. Fish production has observed a steady increase from

3.84 MT in 1991 to 10.06 MT in 2014-15. A steady growth has been shown in marine sector since 2008-09. An inland fishery presently has a share of about 65% in total fish production of the country. The sector contributes about 1% to the overall GDP and around 5.5% of the GDP from Agriculture and allied activities.

1.3 Marine Export

India has a significant role in world's fish and fishery product exports. The major marine products exported were frozen shrimp, frozen fish, frozen cuttlefish, frozen squid, dried items, live items and chilled items. During the financial year 2015-16, India has exported 9, 45,892 MT of Seafood worth US$ 4.7 Billion (Rs. 30,420.83 crores). Frozen shrimp continued to be the major item of export in terms of quantity and value, accounting for a share of 39.53 % in quantity and 66.06% of the total USD earnings. USA continued to be the major importer of Indian seafood with a share of 28.46% in terms of USD followed by South East Asia (24.59%), EU (20.71%) & Japan (8.61%). Vizag, Kochi, JNP, Pipavav and Calcutta are major ports handled the marine cargo (MPEDA, 2016).

Fisheries sector contributes to the national economy providing livelihood to roughly around 14.49 million people in the country. It has been acknowledged as a powerful income and employment generator as it stimulates growth of a number of subsidiary industries. It is also a source of cheap and nutritious food besides being a source of foreign exchange. Fisheries are recognized as a promising sub-sector of agriculture and allied activities in India.

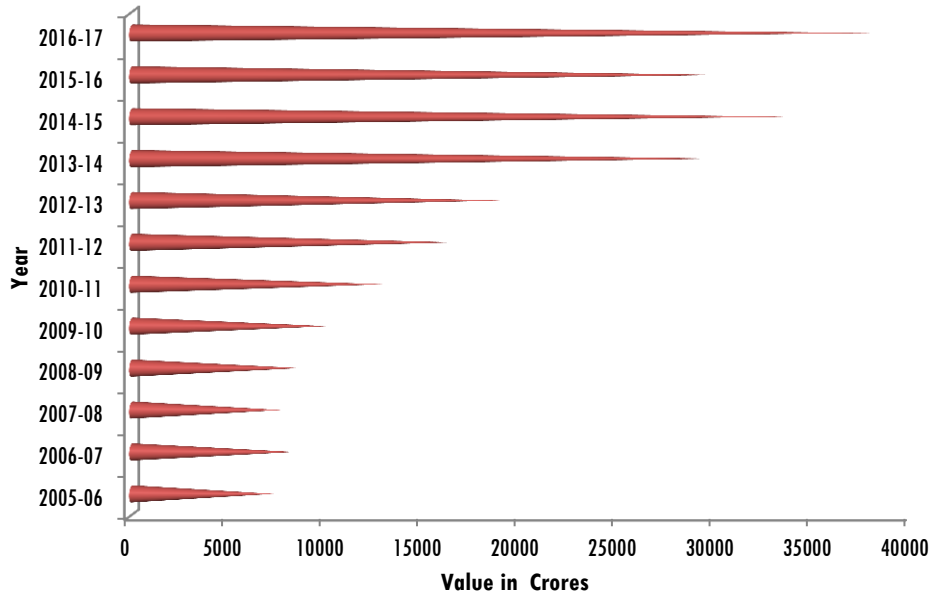

Fig: 1.1 Year wise Export comparison of marine products (MPEDA, 2016)

Table: 1.1 Top marine products export by India (MPEDA, 2016)

	Fr. Shrimp	Fr.Fin Fish	Fr.Cuttle fish	Fr. Squid
Year		**Value In Crore**		
2005-06	4271.51	998.7	549.15	575.52
2006-07	4506.08	1452.88	797.37	568.32
2007-08	3941.62	1303.41	744.13	408.42
2008-09	3779.8	1722.34	761.05	632.35
2009-10	4182.35	2032.33	923.83	622.63
2010-11	5718.13	2623.89	1104.57	1010.57
2011-12	8175.26	3284.15	1346.72	1228.19
2012-13	9706.36	3296.86	1354.28	1378.08
2013-14	19368.3	4294.81	1386.98	1731.97
2014-15	22468.12	3778.5	1833.21	1275.25
2015-16	20045.5	3462.25	1636.11	1615.21

1.4 Characteristics of Cephalopods

Cephalopods, which have gained great grandness in recent years due to the increasing demand in the export trade and their contribution spreads about 3.0 to 3.5% of the whole fish landings of India (Anon, 2001). However, researchers studied that the total world landing of cephalopods stand for only a few percent of the potential resources (Guerra, 1991).Cephalopods namely octopus, cuttlefish and squid - comprise one of the most significant components of marine life. They are the largest invertebrates on earth, most intelligent, most mobile, and the largest of all molluscs. They are a vital part of ocean ecosystem and are heavily exploited as a major human food source.

Their nervous system is developed to a much higher degree than that of other molluscs. They have no skeleton, neither internal nor external. Cephalopod's bodies are divided in head, visceral sac and foot, the mantle (pallium) as a protective coat of the visceral sac. Squid, octopuses, cuttlefish, the chambered nautilus, and their relatives display remarkable diversity in size and lifestyle with adaptations for predation, locomotion, disguise, and communication. They have evolved suckered tentacles, camera-like eyes, color-changing skin, and complex learning behavior.

1.4.1 Squid

The classification of recent taxa of the molluscan Class, Cephalopoda includes the squids which come under the order Teuthoidea, which are further classified based on their eyes. The eyes are covered by a transparent cornea placed in the suborder Myopsida or exposed were grouped in another suborder Oegopsida. The two families of squid, the Ommastrephidae and the Loliginidae, are the major ones of world squid catch. The loliginids are distinguished by a membrane covering the eye, which in Ommastrephids is slit exposing the lens to

the sea. Loliginid squid occur sporadically which are coastal and can only support local fisheries; they yield a higher quality meat than the Ommastrephids. The family Loliginidae includes many varieties that are important in trophic systems, fisheries, environmental and biomedical studies.

Squids are survived mainly in deep water on the Atlantic border of the continental shelf, and to move inshore seasonally or in connection with the breeding cycle. Squid grow quick; Loligo reach sexual maturity 1 year after hatching. The females spawn in the second year, when the mantle reaches a length of up to 18 cm, and then die. The males reach a length of about 30 cm at the end of the first year, and can be 50 cm long when 2 years old. The life span of squid is seldom more than 2-3 years. Squid feed on plankton after hatching, but the adults are active predators which feed on crustaceans, fish and other squid. Although some species float with the ocean currents, the commercially important ones are active swimmers and move speedily through the water by jet force; they contract the thick muscular wall of the mantle and expel water powerfully through the syphon. Squid of the Loligo species have a blunt tail, and the fins or wings together form roughly a diamond shape. The length of the mantle can be up to 60 cm. The squid fishes were catch by a common known method, squid jigging. Squids were attracted to light and they aggregate close to the illuminated area. They are also easily attracted to fast moving bait or bait like object. Squid jigging operation take advantage of this behavior of squids. In India, from January to March and October to December was the most productive period of squid species along the upper east and west coast, while in southern region such as Karnataka, Kerala, Tamil Nadu and Andhra Pradesh equal productivity was observed in July to September.

Table: 1.2. Scientific classifications of Squid

Squid belongs to
Kingdom Animalia
Phylum Mollusca
Class Cephalopoda Cuvier, 1797
Subclass Coleoidea Bather, 1888
'Cohort' Neocoleoidea Haas, 1997
Super order Decapodiformes Young et al., 1998
Order Teuthida Naef, 1916
Suborder Myopsina Orbigny, 1841
Family Loliginidae Lesueur, 1821
Genus Loligoduvauceli Orbigny, 1848
Subgenus Photololigo Natsukari, 1984

1.4.2 Squid Export

Among the cephalopods, squid and cuttle fish contributed to the major export value item accounting for a share of 10.24 % of the total US $ earnings. During the period of 2015-16 frozen squid have shown a growth in terms of quantity as well as in value, and are recorded a growth of 17.54%, 26.66% and 17.96% in terms of quantity, rupee value and USD earnings respectively. USA is the biggest exporterof squid accounting for over 27.8% (Fig 1.2). Developed countries accounted for more than 80% of the total. Japan is the biggest importer accounting for over 25% of the global total. The EC is depending on over 35% of the share for its fish imports.

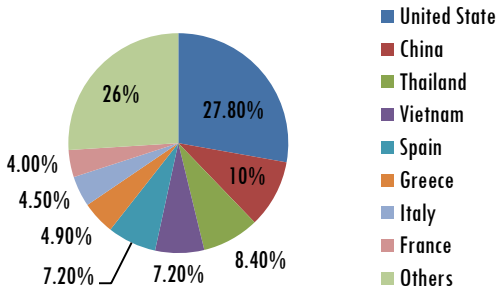

Fig: 1.2 Major squid export country by quantity

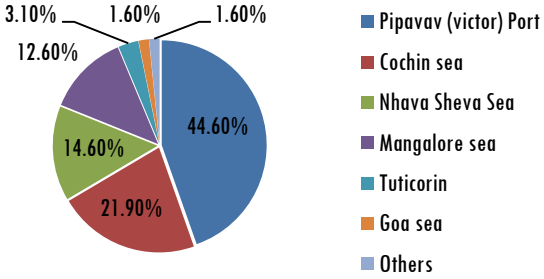

Fig: 1.3 Major Indian port squid export by quantity

1.4.3 Indian Squid (*Loligo duvauceli)*

Among the squids, the Indian squid is the dominant species, catching about 97% from Indian waters. The most common Indian squid distributed in Indo-Pacific ocean periphery, including the Red Sea and the Arabian Sea, extending from Mozambique to the South China and the Philippines Sea, northward to Taiwan. The biology of *Loligo duvauceli* was studied by Silas et al. (1986) and Rao (1988). The report reveals that the males and females are found to be in equal proportion. It matures from the size ranges of 4 to 18 cm in female and 4 to 28 cm for males. Juveniles of squids are of 4 cm in size. Females were leading during January, March, May and December, whereas males were dominant in other months. The overall male to female ratio was 1: 1.3. The size and the morphometric character of the *Loligo* squid varies with geographical locations.

Table: 1.3 Synonyms for *Loligo duvauceli* Orbigny, 1848

Synonyms	Reference
Uroteuthis (Photololigo) duvauceli Rehder, 1945	Roper, et al.,1984
Loligo oshimai Sasaki, 1929	
Loligo indica Pfeffer, 1884	
Loligo galatheae Steenstrup, 1885	Nesis, 1987

Table: 1.4 Common Names for *Loligo duvauceli* Orbigny, 1848

Common Name	Language
Indian squid	English
Calmar indien	French
Calamarindico	Spanish
Bed	Bengali (West Bengal, India)
Samudrasasha	Bengali (West Bengal, India)
Narsingha	Gujarati (Gujarat, India)
Bondas	Kannada (Karnataka, India)
Manki	Konkani (Goa, India)
Mankli	Konkani (Goa, India)
Koonthal	Malayalam (Kerala, India)
Nal	Marathi (Maharashtra, India)
Narsingha	Marathi (Maharashtra, India)
Kalirinda	Oriya (Orissa, India)
Kumitimuna	Oriya (Orissa, India)
Usikanava	Tamil (Tamilnadu, India)
Kandavaya	Telugu (Andhra Pradesh, India)
Kolakalivinda	Telugu (Andhra Pradesh, India)
Kondavai	Telugu (Andhra Pradesh, India)

1.4.4 Squid Handling and Storage

Squid are not normally gutted at sea; they are simply washed and packed in ice. They are more susceptible to damage than gutted white squid if not handled carefully; crushing, scuffing or tearing of the skin, and burst ink

sacs are indicative of rough handling. Squids are kept ungutted because many places prefer them whole, the ink and the tentacles are often used along with the flesh during cooking. Stowage in boxes is normally better than bulk stowage because there is less risk of bursting the ink sac. There should be at least 1 portion of ice to 3 portions of squid by weight. Gutted cleaned squid in ice keep in first class condition for up to 8 days. Un-gutted squid stored in chilled sea water keep in good condition for 6 days, and become inedible after 9 days. Whole squid can be marketed in ice or frozen. Fresh squid should be packed in cartons and frozen quickly. An air blast freezer is suitable and can be stored at - 30°C for 9 months or more.

The squid are prepared by the whole squid is washed, and the tentacles are cut off just in front of the eyes or these are retained once the suckers have been removed. The head is twisted and the mantle is squeezed whilst the head, pen and guts arc gently pulled out. The mantle cavity washed out, or it can be split and opened so that any remaining guts can be scraped or washed away. The skin on the mantle can be peeled off; blanching in hot water at 25-30°C for about 15 seconds makes the skin easier to remove. Machines for heading, gutting, peeling and cutting squid is available. The yield of edible flesh from squid, 60-80 per cent including mantle, fins and tentacles, is higher than that from white fish. The only rigid parts are the beak, the cartilaginous pen and the rings of cartilage in the suckers.

A major share of Loligo species landed in India is exported as whole Loligo and only a small portion is sold in local markets. Thus there is little incentive towards development of new squid products in the market but outlets possibly worth pursuing include frozen packs of strips or rings of squid enrobed in batter, and paella or other seafood dishes containing pieces of squid meat.

Table:1.5 Squid products exported from India to various countries (Anusha et al., 2014)

Items exported	Origin	Market
Fillet	Tuticorin	Japan
Wings	Tuticorin	Japan
Whole (Cleaned)	Kollam and Veraval	USA & European Union
Whole	Kollam and Mangalore	Spain & UAE
Whole (Cleaned)	Mumbai	Italy
Rings blanched IQF	Kochi	Italy & France
Tentacles blanched IQF	Kochi	Italy

1.4.5 Squid Safety and Quality

Food safety and food quality are important issues nowadays in world. Squid is one of the highly perishable food items and it is very important to process and preserve in order to guarantee safe and fresh product. Advances in food technology helped to curtail opportunities for chemical, microbiological hazards and significant developments in laboratory diagnosis such as the novel techniques developed.

For commercialization, it is essential to estimate its freshness, one of the most important quality aspects of squid and squid products. Thus, there is a need for rapid analytical techniques for analyzing the quality, safety and freshness of squid and its products.

Sensory evaluation is one of the most important method for assessing freshness and quality of seafood. Sensory evaluation is a rapid and accurate tool for providing unique information about food, however it is subject specific. The compounds formed during fish spoilage have been frequently used to assess the quality of different species. Quality indices are mainly based on analyzing the concentration of protein or nucleotide degradation products. Different freshness or spoilage index have been established for fish as indicators of decomposition or as an indicators of hygiene and handling. However, many of these chemical indices are developed mainly for fish, not

for the cephalopods especially for squid. As food safety management moves towards more risk and evidence based approaches, there is a need to develop a new quality index for squid.

Biogenic amines are non volatile nitrogenous compounds of biological importance in vegetable, animal and microbial cells. They can be identified in both raw and processed foods. In food safety their accumulation sometimes been related to freshness index, spoilage and fermentation processes. Some toxicological characteristics and outbreaks of food poisoning are associated with biogenic amines. The presence of biogenic amines above a certain level is considered as indicative of undesired microbial growth. Biogenic amines are important, irrespective of their health significance, and can serve as freshness indicator in different fishes like Tuna, Sardine Mackerel, Rockfish, Salmon, Lobster, Shrimp etc. The aim of the study was to characterize the chemical, sensory, and microbiological changes of Indian squid during different storage conditions and to determine the usefulness of some available methods especially biogenic amine for quality evaluation

1.5 Objectives of the Study

- To study the chemical composition of Indian squid and highlight the aminoacid composition
- To study the quality changes of whole and gutted squid in iced conditions with special emphasis on biogenic amines changes
- To study the effect of delayed icing on biogenic amines formation in Indian Squid
- To study the effect of different storage temperature and time on the biogenic amine, microbial sensory and other chemical developments in Squid
- To develop Biogenic amine as a quality index in Indian squid
- To recommend effective process control measures to increase the utilization of the species

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Chapter

2

REVIEW OF LITERATURE

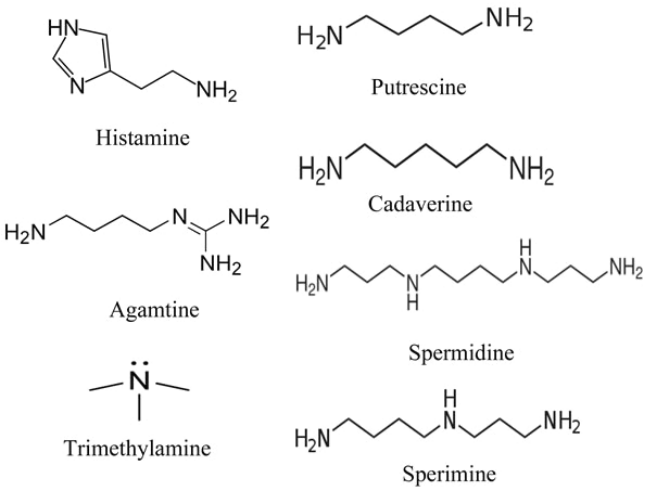
Contents

2.1 Biogenic amines

Biogenic amines are non volatile, low molecular weight nitrogenous organic compounds formed by specific reactions involving aminoacids. They are produced in the human body by amination of aldehydes and ketones (Ababouch et al., 1991; Halasz et al., 1994; Shalaby et al., 1996; Karovicova and Kohajdova 2005; Lorenzo et al., 2007; Ismail et al., 2009; Aishath et al., 2010). Biogenic amines are produced in foods by the action of certain strains of bacteria through the decarboxylation of aminoacids. (Shalaby 1996; Suzzi and Gardini 2003; Rivas et al., 2008). Based on their structure biogenic amines are classified as aliphatic, aromatic, heterocyclic amines includes putrescine,

cadaverine, spermine, spermidine, tyramine, 2-phenylethylamine, histamine, tryptamine, agmatine, octopamine and dopamine. They can also be classified as monoamines (phenyl ethylamine, and tyramine) diamines (cadaverine, putresine) and poly amines (spermidine, spermine) based on the number of amine group. Polyamines are naturally present in the food. (Spano et al., 2010; Hernandez et al., 1996; Hornero et al., 1997; Kim et al., 2009). Histamine, cadaverine, putrescine, agmatine, tyramine beta-phenyl ethylamine are produced by decarboxylation of histidine, lysine, ornithine, arginin, tyrosine, phenyl ethyl alanine respectively. In plants and some micro organisms, alternative pathway exists to produce putrescine from arginine via agmatine and cadaverine is also produced by the ornitine decarboxylase enzyme from ornitine. (Basavakumar et al., 1992; Beutling et al., 1996; Lovaas.,1991)

2.1.1 Structure of Biogenic amine



2.1.2 Functions of amines in human body

Amines are formed during normal metabolic process in all living organisms; however the characteristics and biological functions of amines are very diverse. Dietary amines are traditionally known as natural poly amines

(putrecine, spermidine, and spermine) or biogenic amines based on their synthesis. Natural polyamines are formed during *de novo* poly amine synthesis, while biogenic amines are formed by non- specific de-carboxylation reaction. (Susan bardoz., 1995). Poly amines are essential for keeping high metabolic activity of the normal functioning and immunological systems of the gut. Poly amines are also play an important roles in synthesis of protein, nucleic acids, stimulate ribosome subunit association, stabilize the t-RNA structure, reduce the rate of RNA degradation, regulate the rigidity and stability of cellular membrane and the synthesis of hormone (Silla santos., 1996; Susan bardoz., 1995). They can also influence the processes in the organism such as regulation of body temperature, intake of nutrition, increase or decrease of blood pressure (Grief et al., 1997). Due to the diversity of their roles in cellular metabolism and growth, they are required in rapidly growing tissues in large amounts (Bardoz., 1989). Although they have long been known to be essential for growth, but their exact biological role in cell metabolism is still uncertain.

Certain classes of amines, the catecholamines, indolamine fulfill an important metabolic function in the humans, especially in the nervous system and the control of blood pressure (Halasz et al., 1994). Histamine reduces blood pressure; by contrast phenyl ethylamine and tyramine cause a rise in blood pressure. Histamine possesses a powerful biological function, involved as a primary mediator of the immediate symptoms noted in allergic reactions (Statton et al., 1991). Other vital role in histamine include wound healing, mediation of vascular permeability and mucus secretion, day-night rhythm, hepatopoiesis, angiogenisis in tumor models, immunomodulation, (Kusche et al., 1980; Raithel et al., 1998), and the control of amine- induced cell proliferation intestinal ischemia (Kalchmair et al., 2003). Histamine also

many vital functions in healthy individual ranging from manage of gastric acid secretion to neurological transmission in the central nervous system (Maintz and Novak, 2007; Katzung, 2007). For many, the most common evident response to histamine involves the immune system and, especially, allergic responses (White, 1990). Spermine and spermidine have been implicated in the evolution of intestinal tissue. (Romain et al., 1992; Bardsz 1993). Putrescine, cadaverine, and spermidine etc have been reported as radical scavengers (sillasantos, 1996). It has reported that the biogenic amines delay the oxidation of poly unsaturated fatty acids, and this function is correlated with the number of amine groups in the poly amine (Lovaas, 1991). It is demonstrated that tyramine has an antioxidant effect may be depends on its hydroxyl groups and amines. In plants and trees, putescine, spermidine and spermine are involved in different physiological functions, such as cell growth, flowering, fruit development and maturation, response to stress and senescence etc. (Halaz et al., 1994).

2.1.3 Types of Biogenic amines

Histamine

Histamine is a powerful biologically active organic nitrogenous compound involved in regulating physiological function in the gut as well as local immune responses and also served as a neurotransmitter (Marieb, 2001). Histamine is produced in the body as a result of decarboxilation of aminoacid histidine, the reaction catalysed by the enzyme L-histidine decarboxylase. It is a hydrophilic vasoactive amine. Histamine is produced in to the blood stream as an inflammatory response and act as mediator of pruritus (Andersen et al., 2015). As the function of an immune response to foreign body, histamine is formed from basophils and by mast cells found in nearby connective tissues.

Histamine increases the permeability of the capillaries and to affect the functions of white blood cells and some proteins. (Di Giuseppe et al., 2003). Once formed in human body histamine is either stored or rapidly inactivated by ezymes. Histamine produced in the synapses of central nervous system is primarily decomposed by histamine-N-methyltransferase, while in other tissue enzymes histamine-N-methyl transferase or diamine oxidase may play a major role. Other enzymes, including MAO-B and ALDH2, further break down the immediate metabolites of histamine for recycling or excretion.

Bacteria are also capable of producing histamine in food with the help of enzymes like histidine decarboxylase, unrelated to those found in human and animals. Scombroid poisoning, a non-infectious form of food born disease is due to histamine production by bacteria in spoiled food, particularly scombroid fishes. Beverages and fermented food products naturally contain small amount of histamine due to similar degradation performed by fermenting bacteria or yeasts. Histamine-rich foods may cause food intolerance in sensitive individuals and histamine contamination in fish and fish products may cause food poisonings (Taylor, 1986)

Putrescine

Putrescine is a polyamine first isolated from spoiled meat. Putrescine or tetramethylenediamine is a foul smelling organic chemical compound, produced by the breakdown of amino acids ornithine in spoiled foods (Haglund et al., 1996). Putrescine is produced in healthy living cells in small amount by the action of ornithine decarboxylase to ornithine. In living and organism putrescine are toxic in large doses (Kamhi et al., 2007; Lews et al., 1998). In laboratory experiments rats shows a fairly low acute oral toxicity of 83mg/kg body weight. In the living cells decarboxylated S-adenosyl

methionine attacks on produced putrescine and gets converted to spermidine. Spermidine in turn attacked by another decarboxylated S-adenosyl methionine and gets converted to spermine. In human body the polyamine, putrescine may act as growth factor necessary for cell division. Putescine presence with aminoacid cadverine has been found to potentiate histamine allergy in fish and meat. Putrescine has been mainly studied as a spoilage indicator in many food items. (Kalac et al., 2005).

Cadaverine

Cadaverine is a foul smelling, organic, nitrogenous compound produced from amino acid lysine during bacterial spoilage or fermentation and is toxic in large doses (Lewis et al., 1998). Cadaverine is also known by the name 1, 5-pentamethylenediamine. In rats it had an acute oral toxicity of 83 mg/Kg body weight (Til et al., 1997). In some species of fish the cadaverine is formed earlier than the histamine during storage. (Pons Sanchez, et al., 2005; Rosy et al., 2002). Like putrescine, cadaverine is also acts as histamine potentiators. (Taylor and Lieber, 1979).

Tyramine

Tyramine is a naturally occurring aromatic monoamine compound derived from the amino acid tyrosine. Tyramine occurs widely in plants and animals (Smith, 1993) and is metabolized by the enzyme monoamine oxidase. Large amount of tyramine found in spoiled, pickled or fermented fish (Leuschner and Hammes., 1999; Prester, 2011). Like Putrascine and cadaverine, tyramine acts as a histamine potentiator (Taylor and laber., 1979). 'Cheese reaction', is an important hypertensive crisis followed by the consumption of food rich in tyramine (Hernadez-jovar et al., 1997; Bodmer et

al., 1999; Alberto et al., 2002; Ruiz-Capillas and Jimenez-Colmeneo, 2004; Karovicova., 2005; Onal 2007).

Other biogenic amines

Other biogenic amines includes agmatine, tryptamine, spermine, spermidine are formed from free aminoacids namely arginine, tryptophane, putrescine (Zarei et al., 2011). The free aminoacids may be occur in the food as such or liberated through protein hydrolysis are involved in the formation of biogenic amine.

2.1.4 Biogenic amines in foods

In most of all plants and animal based food products contain proteins or free amino acids and are subject to conditions enabling microbiological or biochemical activity, biogenic amines can be expected. (Silla Santos, 1996; Halasz A, et al., 1994). Biogenic amine is also present in wide range of fermented foods including sausages, fermented dairy products, meat products, fermented vegetables, wine, beer etc (Askar and Treptow, 1986; Brink, et al., 1990).

Fish and fish products

Major biogenic amines includes histamine, putrescine, cadverine, agmatines etc are very significant in fish and fish products in safety and quality point of view. Biogenic amines are formed at low levels in fresh fish and their production is related to the bacterial spoilage (Ozogul&Ozogul, 2006). Among the biogenic amines histamine is the major one since they have contributing common fish poisoning known as histamine poisoning. Biogenic amines such as putrescine and cadaverine can potentiate the toxicity of histamine through interfering with histamine detoxification system. Fishes contain red muscles which are rich in free histidine varying from 1g/Kg in

herring to as much as 15g/kg in tuna and they can be catabolysed by two ways in fish muscle. The histidine deamination to obtain uronic acid or the histidine decaroxylation to produce histamine (Rondriguez-Jerez et al., 1994; Shakila et al., 2003; Onal, 2007). The deamination activity is the main way in normal physiological condition; while the decarboxylation activity is most important in bacterial contamination (Vidal and Marine, 1984). Histamine food poisoning is also known as scombroid fish poisoning (scombrotoxicosis) (Morrow et al., 1991;Onal, 2007). The name 'scombroid' is derived from the family *scombridae* which includes the fish species such as tuna and mackerel that were first implicated in histamine intoxication. Scombroid fishes contain high levels of free histidine in their muscle tissues. Several studies of histamine production in different marine fishes were conducted by researchers. (Baranowski et al., 1985; Klausen and Huss, 1987; Halaz et al., 1994; Valeiro and Pilas, 1994). It is also noted that some of the non scombroid fish species such as mahi-mahi (*Coryphaena spp*.) (Frank et al., 1985), anchovies (*Engraulis spp*.), sardine (*Sardinella spp*.) (Ababouch et al., 1991) herring (*Clupea spp*.), pilchards (*Sardina pilchardus spp*.), marlin (*Makaira spp*.), Western Australian salmon (*Arripis truttaceus*), blue fish (*Pomatomus spp*.) (Karolus et al., 1985), swordfish (*Xiphias gladius*), cape yellow fin (*Seriola lalandii*) etc. are implicated in scombroid poisoning. Most of these fish species rich in free histidine except sword fish and salmon.

Meat and meat products

Biogenic amine in meat and meat products have been reported to contain tyramine, cadaverine, putrescine, spermine, and spermidine (Koehler and Eitenmiller, 1978; Nakamura et al., 1979; Edwards et al., 1983; Santos-Buelga et al., 1986; Stratton et al., 1991; Bamkz, 1993; Shalaby, 1993, 1994, 1995). In cooked and uncooked pork meat and in ground beef various biogenic

amines have been detected (Nemeth-Szerdahelyi et al., 1993). Straub, (1994) reported large amounts of cadaverine present in beef have been associated with heavy contamination by *Enterobacteriaceae*. Biogenic amines were also identified in fresh vacuum-packaged beef during storage at 1°C for 120 days; significant levels were detected at day 20. It was also reported that although cold stored vacuum-packaged beef was sensorily acceptable, it could create some risk to individuals sensitive to biogenic amines (Smith et al., 1993). Fresh and processed pork meat contains high levels of adrenaline, spermidine spermine and traces of noradrenaline, putrescine, histamine, cadaverine and tyramine (Halsz et al., 1994). It was reported that increases in the biogenic amine content of pork are temperature dependent. In most of the studies, biogenic amine concentration increases during storage, while both spermine and spermidine concentration decreases. Pork stored at 30°C had higher biogenic amine levels than pork stored at 4°C while biogenic amine levels of pork stored at -18°C did not alter over a month period (Chen et al., 1994). Concentrations of putrescine, cadaverine, and histamine were significantly associated with the volatile basic-N (VB-N) values of pork and it intern correlated with the levels of spoilage (Nemeth-Szerdahelyi et al., 1993), therefore, freshness of pork can be evaluated accurately by the amine content combined with VB-N (Chen et al., 1994).

Histamine and the factors depends on its synthesis have been determined in sheep, bovine muscle and in other kinds of meat and meat products. (Teodorovi et al., 1994; Santos et al., 1985). Meat from harp seal *(Phoca groenlandica)* has high protein content with well-balanced essential amino acids (Shahidi et al., 1990). Harp seal is also containing high amount of non-protein nitrogen compounds such as amines, including spermine, spermidine and ethanolamine (Shahidi et al., 1993).

The occurrence of biogenic amine in fermented meat sausages may originate from contaminated raw material or from the fermentation process itself. Edward et al, (1987) reported that the tyramine formation in vacuum-packed meat was caused by *Carnobacterium divergens* and the formation of putrescine and cadaverine was found responsible by *Enterobacteriaceae* or strains of *Pseudomonas*. During the sausage ripening process, the histamine concentration increases at least ten times during the initial days of storage ripening (Dierick et al., 1974). In common, quite variable quantities of biogenic amines were reported in sausages. The variable concentration of biogenic amine could be due to the variation of the ripening time, the type and quality of the meat used, manufacturing process, and difference of decarboxylase activity of the natural microflora responsible for fermentation (Askar, 1979; Shalaby and Abd El-Rahman, 1995; Shalaby, 1995).

Milk and milk products

Biogenic amines are present in wide range milk and milk products, and can accumulate in high concentrations. In some cheeses more than 1000 mg of biogenic amine have been detected per kilogram of cheese (Linares, 2011). In full cream and semi-skimmed cow's milk low amounts of polyamines have been detected (Bardcz et al., 1993). Kolesarova, E (1995) reported that the occurrence of biogenic amine in the milk is low, about 1 mg dm^{-3}, but in the cheese their content achieves 1 g kg^{-1}. Biogenic amines have been found in small amounts in dried milk (Voigt et al., 1974). Other milk products made from pasteurized milk such as kefir and yogurt have little or no detectable levels of tyramine. A recent study compared the amino acids profiles of milk, kefir, and yogurt found few differences suggesting comparable risks of biogenic amine formation (Guzel-Seydim et al., 2003). In human milk biogenic amines have been detected and showed a pronounced variation in the

quantity of spermine and spermidine between the left and right breast (Romain et al., 1992).

Cheese is the second most commonly implicated food item associated with histamine poisoning after fish and the first reported case was in 1967 in the Netherlands in Gouda cheese (Stratton et al., 1991). Many studies have been undertaken to determine the amine content of different cheese products. Different amines such as histamine, cadaverine, putrescine, tryptamine, tyramine and phenylethylamine have been detected in many types of cheeses (Stratton et al., 1991; Tawtik et al., 1992; Besanson et al., 1992; Celano et al., 1992; Diaz et al., 1992; Moret et al., 1992: Spanjer and Van Roode, 1991; El-Sayed, 1997; Novella-Rodriguez et al., 2002; Durlu-Ozkaya, 2002; McCabe et al., 2003.). Histamine and tyramine present in varying levels extensively not only between different types of cheese but within the varieties (Stratton et al., 1991). Cheese contains proteins, enzymes, cofactors, water, salt, and bacteria, and therefore represents an ideal condition for biogenic amine production from free amino acids by decarboxylating enzymes of microorganisms during cheese ripening (Rodriquez, 2000). During cheese ripening, protein casein is slowly breakdown by proteolytic enzymes, leading to an increase of free amino acids concentration (Foster et al., 1958; Joosten and Olieman, 1986). These amino acids can be subjected to subsequent degraded reactions and catalysed by specific bacterial decarboxylases enzymes to give rise to the formation of CO_2 and an amine (Degheidi et al., 1992). Large amounts of biogenic amine in cheese could indicate a failure from a hygienic point of view, in the milk used for cheese products or during the cheese making (Masson, 1996).

Fruits and vegetables

Amines are commonly detected in fruits and vegetables as an endogenous substance (Lovenberg, 1973). Biogenic amines in plant substances give the typical and characteristic taste of mature foods and are precursors of certain aroma compounds. Surprisingly, not many investigators have extensively analyzed fruits for their amine content, and the data indicate that same varieties of the fruit have varying amine levels (Maga, 1978). Halsz et al., (1994) have reported high amine levels in tomato (tyramine, tryptamine, and histamine), banana (tyramine, noradrenaline, tryptamine, and serotonin), spinach leaves (histamine) and plums (tyramine, noradrenaline). Some species of mushrooms contain high concentration of phenylethylamine (Pfundstein et al., 1991). Biogenic amine levels in straw mushrooms (*Volvariella volvacea*) were found to increase with storage time at both 4°C and 25°C but reduction by about 80% was obtained after boiling for 5 min. Yen (1992) reported canned mushrooms were contain a total of 7.1 mg/kg of putrescine, tyramine and 1-phenylethylamine. Lettuce, endive, Chinese cabbage, ice-berg and radicchio were found to contain amines with concentration ranging from 14 to 20 pg g^{-1}, spermidine was found to be the major polyamine presented in these vegetables at a concentration of 7-15 pg g^{-1} (Simon- Sarkadi and Holzapfel, 1994).

Reports on the hygienic status of leafy vegetables indicate the association of high microbial numbers and the presence of biogenic amine, both with the fresh and packed products (Antolini, 1999). Phenylethylamine is naturally present in cocoa beans and thus occurs in chocolate, chocolate products and confectionery containing chocolate. Histamine and cadaverine have been found in carrageenan from algae (Barwell, 1994). In white and black pepper high levels of pyrrolidine have been detected (Pfundstein et al., 1991). Maxa and Brandes (1993) reported that putrescine was the major amine

in the majority fruit juice samples including lemons, raspberries, mandarins, grapefruit, currants strawberries, and grapes. Histamine was little in these juices, except in a lemon juice sample. Raspberry juice contain high tyramine concentration of 66.66 mg 1^{-1}. Halsz et al., (1994) have reported that high amine levels in orange juice (noradrenaline, tryptamine).

Fermented vegetables represent another major class of foods from which biogenic amines have been isolated. Low amounts of biogenic amines could be found in fermented vegetables following inoculation with *L. plantarum* (Andersson, 1988). Sauerkraut is a fermented cabbage produced by the action of different lactic acid bacteria producing biogenic amines includes *Lactobacillus sp*., producing putrescine and tyramine, *Pediococcus cerevisiae*, producing histamine in content about 200 mg/kg and *Leuconostoc mesenteroides*, producing putrescine of about 250 mg/kg. (Halasz, 1999; Hornero-Mendez, 1997; Kuensh et al., 1989). Joosten and Olieman (1986) indicated that a sauerkraut extract contained a large amount of putrescine 210 mg/kg, as well as cadaverine tyramine, and histamine were detected with concentrations of 79, 69, 58 mg/kg, respectively. The average histamine level of the retail sauerkraut samples was 51 mg/ kg (Taylor et al., 1978a). Although sauerkraut routinely checked levels of histamine far below the toxic dose of 1 g /kg (Stratton et al., 1991), it has been reported histamine poisoning in Europe indicating that sauerkraut has been detected to occasionally contain amounts of histamine near the toxic dose (Mower and Bhagavan, 1989). Sauerkraut fermentation studies (Kuensh et al., 1989) found that considerable amounts of putrescine (150 mg/kg) were formed during the initial period of fermentation, on other hand arginine shows decrease in concentration, while histamine and tyramine appeared at the final stage of fermentation. Accordingly, the production of sauerkraut with low in histamine can be

achieved by minimizing the fermentation period. The biogenic amine, especially putrescine, accumulates in sauerkraut brine (Bardocz, 1995). The cadaverine, histamine, putrescine, spermidine, and tyramine were found in the lactic acid fermented vegetables such as carrot and red beet in content ranging from 1 to 15 mg/kg (Simon-Sarkadi, L., 1994).

Soy products

Soy products includes soy souse, miso, and tempe, soy curd, soy paste etc are very common in Western countries. Since different varieties micro organisms such as molds, yeasts, and lactic acid bacteria are involved in the fermentation processes of soy products and the raw materials includes soy beans, legumes, cereals or other materials contain significant amounts of protein, the formation of different amines might be predictable during the fermentation process (Chin and Koehler., 1983). Histamine and tyramine have been detected at various levels of fermentation in such soy products (Stratton et al., 1991). Increased level of biogenic amines (1g/kg) was detected in soy sauce made from black soy bean (Yen, 1986). Among the biogenic amines tyramine has been detected at moderately high levels (450 mg/kg) in fermented salted black soy beans (Mower and Bhagavan, 1989). The difference in biogenic amines levels in the commercial fermented soy products depends on the used raw material, the microbiological composition and the conditions and duration of fermentation (Chin and Koehler, 1983; Nout et al., 1993). Nout et al., (1993) reported that the functional fungus *Rhizopus oligosporus*, mainly produced tyramine and some putrescine in tempe, but *Lactobacillus plantarum* reduced tyramine levels in that product while both *Trichosporonbeiglli* and *Klebsiella pneumonia* led to increased total biogenic amines in the tempe. In meso, a Japanese fermented soybean paste, histamine decarboxilase bacteria have been identified as *lactobacillus* species (Ibe et al., 1992).

Wine &Beer

More than 20 biogenic amines have been studied in fermented beverages especially in wine and their level has been reported to range from a few mg/l to 50mg/l (Lonvaud-Funel, 2001; Landete et al., 2005). In Swedish, Danish, and French beers histamine was detected ranging from 4.7 to 20 mg/l (Zee and Simard, 1981). Tyramine was also found by levels as high as 11 mg/l in beers of various origins (Maga, 1978). Stratton et al., (1991) reported that putrescine and cadaverine concentrations are generally low in beers. Agmatine, putrescine, and tyramine were detected at concentrations ranging from 0.55 to 67.60 mg/l, at the same time histamine, cadaverine, tryptamine, spermine, spermidine, and p-phenylethylamine shows concentration lower than 2 mg/l, but in some European beers relatively high levels of histamine and cadaverine were detected (Izquierdo-Pulido et al., 1994). Some authors reported the presence biogenic amines in beers can be related to the microbial contaminations in raw materials or to during brewing but not for the brewer's yeast, since brewer's yeast appear to lack the ability for producing amines during fermentation,

Shalaby, A. R (1996) reported that the presence of biogenic amines in wines may be from the must, or formed by the action of yeasts or as result of bacterial action involved during malolactic fermentation. Predominant biogenic amines in wine are histamine, tyramine, putrescine, isophenylamine, and β- phenylethylamine. Maximum levels biogenic amines in wines from different European origin were reported as high as 16.6 mg dm^{-3} histamine, 20.2 mg dm^{-3} tyramine, and 76 mg dm^{-3} putrescine. Mean levels of histamine were at 3.63 mg dm^{-3} for French wines, 2.19 mg dm^{-3} for Italian wines, and 5.02 mg dm^{-3} for Spanish wines (Beutling, 1996).

2.1.5 Factors affecting Biogenic Amines formation

Concentrations of biogenic amines are generally low in fresh foods and the amines levels reaches to toxic concentration during storage especially histamine. Growth and the associated formation of biogenic amines by amines forming bacteria in seafood depend on several environmental factors (Ryser et al., 1984; Tabor and Tabor, 1985; Kim et al., 2000; Lorca et al., 2001; Kim et al., 2001a; Guizani et al., 2005) including availability of substrate (Maijala and others 1995a), storage conditions (Komprda and others 2001), manufacturing techniques and practices (Rivas and others 2008, Komprda and others 2001), raw material quality (Maijala and others 1995b) microbial population with decarboxylase activity (Santos 1996), pH, antimicrobial agents, composition of the storage atmosphere (Taylor 1986; Lehane and Olley 2000) etc. To facilitate the control of biogenic amine production it is important to know the effect of these parameters on the production of biogenic amine. Factors that influence the rate of biogenic amines formation in seafood are discussed below.

pH

pH is one of the major factor influencing amino acid decarboxylase activity of micro organisms that intern affecting the formation of biogenic amine in a medium. Amino acid decarboxylase activity was higher in an acidic environment, being the optimum pH between 4.0 and 5.5 (Teodorovie et al., 1994; Halasz, A., 1994; Marcobal et al., 2006c; Fernandez et al., 2007b; Pons-Sanchez-Cascado et al.,2005a). In such environment organisms produce decarboxylase enzymes as a part of their protection mechanisms against the acidity (Maijala, R.1993: Buneic et al., 1993; Teodorovic et al., 1994 Moreno-Arribas et al., 2001). It was reported that the acidic pH induces structural changes in histidine decarboxylase that increases the activity (Schelp et al.,

2001). It is also found that an increase in production of biogenic amine is related to the induced expression of decarboxylases and transporter genes (Linares et al., 2011). Although Gardini et al. (2001) reported that rapid acidification can lead to reduced biogenic amine production via a decrease in the growth of contaminant gram negative decarboxylating microorganisms.

It has been studied that acidic environments favor tyramine production. Santos et al., (1986a) found a higher tyramine level in mackerel when the pH was low. Pogorzelski, (1992) reported that the histamine concentration in wine was higher with a pH above 3.77. The optimum level for tyramine synthesis in cheese is pH 5.0 (Diaz et al., 1992). *Enterococcus durans*, biogenic amine-producing strain isolated from cheese has produced higher level of tyramine in an optimum pH 5.0 (Fernandez et al., 2006). Baranowski et al., 1985 found that the histamine produced by *Klebsiella pneumoniae UH-2* isolated from skipjack tuna was grown at optimum pH 4.0, with 70% of this activity maintained at pH 6.0. The pH for the formation of the highest concentration of nitrosamines has been reported to be 2.5 to 3.5 (Scanlan, 1983) and 3.8 (Warthesen et al., 1975). It was concluded that low pH enhances nitrosamines formation in fish products, while salted herring contained 0.91 and 0.42 μg/kg respectively (Yurchenko and Molder, 2006).

Temperature

Temperature control has been recommended as a key factor to prevent biogenic amine formation in fish products during handling, processing and storage (Stratton et al., 1991). Number of reports has shown that with respect to the storage temperature influence the production of biogenic amines in food products (Pinho et al., 2001;Gardini et al., 2001; Gennaro et al., 2003; Guizani et al. 2005; Martuscelli et al., 2005; Carelli, D.,2007; Bunkova et al., 2010).

Santos et al., (1986a) found that storage refrigeration temperatures did not significantly influence maximum tyramine content in anchovies. Disagreeing with the above information, Santos et al. (2003) observed a reduction in biogenic amine formation in cheeses made at 20°C instead of 32°C. Diaz et al., (1992) reported that tyramine and histamine concentrations increased in chihuahua cheese with the time and storage temperature. Biogenic amine is produced in raw fish by the action of bacterial decarboxylase enzyme following temperature/time abuse. Production of histamine is greater at high storage temperatures (21.1°C) than at moderate temperatures (7.2°C), whereas its production is particularly rapid at temperatures near 32.2°C (FDA, 2011). Rossano et al. (2006) explained the influence of freezing temperature and time on histamine production in anchovies and found that the ability of frozen temperature to inhibit or retard its formation. Putrescine production by *Enterobacter cloacae* was identified at 20°C after 24 hours of incubation but not at 10°C, and *Klebsiella pneumoniae* showed less extensive cadaverine biosynthesis at 10°C than at 20°C. In foods, histamine production is slowed at 10°C and nearly inhibited at 5°C due to the slow growth of histamine decarboxylase enzyme-producing bacteria at low temperatures. No histamine was produced by *Pseudomonas vulgaris, Pseudomonas morganis, or Hajizia* strains after one month of incubation at 1 °C (Halsz et al., 1994). Similarly, Klausen and Lund, (1986) reported that amine contents were temperature dependent and at 10°C were two to 20 times higher than at 2°C in both mackerel and herring. Studies showed that biosynthesis of putrescine, cadaverine, spermine, spermidine, tyramine and 1, 3-diaminopropane, were all positively interrelated with both storage time and temperature (Sayem-El-Daher et al., 1984). Baranowski et al., (1985) identified that *Klebsiella pneumoniae* UH-2 produces high level of histamine at 10, 25 and 37°C.

Ababouch et al., (1991) reported that maintaining at low storage temperatures was not sufficient to prevent the formation of toxic amines such as histamine. During the storage of pork meat, the concentration of spermidine and spermine decreased at both 5°C and at -20°C, where as the putrescine and cadaverine increased in the same conditions (Halsz et al., 1994). Sayem-El-Daher et al., 1984 concluded that histamine in raw and cooked ground beef levels were unaffected by 12 days storage conditions at 4, 7 and 10°C. Amine concentrations were also unchanged by cooking temperature, with the exception of spermine, which decreased during heat treatment of cooked ground beef at 200°C for 2 h. Luten et al., (1992) and Wendakoon and Sakaguchi, (1993) were also reported that histamine is thermally stable during the cooking. Once these amines are synthesized, it is very difficult to remove them. Nevertheless, the original concentration of amines in food can vary as a result of storage conditions and these should be controlled. According to European Regulation raw, fresh, thawed, unprocessed or processed fishery products from crustaceans and mollusks must be maintained at low temperature (European Commission, 2004). Rapid chilling fish immediately after death is the most important way for preventing the formation of biogenic amine, especially for fish that is grow in warm waters. Failure to chill onboard may allow bacteria to grow and synthesis enzymes, resulting high level amines (FDA, 2011). Many researchers have studied the effects of storage temperatures on biogenic amine formation in fish and their results have been very often confusing (Pinho et al., 2001; Gardini et al., 2001; Gennaro et al., 2003; Guizani et al. 2005; Martuscelli et al., 2005; Carelli, D.,2007; Bunkova et al., 2010). This can be clarified by the varying composition and the level of microorganisms in the fish.

Additives

Naturally occurring additives and artificial chemical additives have shown to control biogenic amine formation (Komprda et al 2004). Natural additives include curcumin in turmeric, capsaicin in red pepper, and piperine in black pepper (Bhutani and others 2009; Shakila and others 1995; Wendakoon and Sakaguchi 1992). Thymol is a phenolic monoterpene, naturally found in essential oil of spices may delay biogenic amine formation in foods (Singh et al., 1999; Lee and others 2008). Ginger, red pepper, garlic, clove, green onion, and cinnamon have been shown to inhibit biogenic amine production in Myeolchijeot, a salted and fermented anchovy (Mah and others 2009). The addition of 5% garlic during ripening of Myeolchi-jeot reduced the biogenic amine level by 8.7% (Mah et al 2009). The 6-gingerol, pungent chemical of ginger (Young and others 2005), delay biogenic amine formation (Singh et al 1999). Ethanol extracts of allspice, cloves, sage, nutmeg and cinnamon, were shown some destroying effect on by *Enterobacter aerogenes*. Wendakoon and Sakaguchi (1995) reported Cinnamic aldehyde and eugenol, an active component of cinnamon and cloves were preventing the production biogenic amine by specific bacteria, *E. aerogenes*. Histamine formation by *M. morganii* was reduced in the presence of essential oil of lemongrass (Shalaby 1996) and by 0.5% potassium sorbate (Sangcharoen and others 2009). Additive glycine 10% was shown to lower the histamine, cadaverine, and putrescine by 93, 78, and 32%, respectively, and decrease spermidine and tyramine production by 100% (Mah and Hwang 2009a). The disadvantage of these chemical components is the considerable loss in efficacy that occurs during heat processing (Suresh and others 2007). Among these substances, capsaicin was found more stable during high temperature processing than

curcumin and piperine (Srinivasan et al., 1992) but, capsaicin is a pungent constituent and stimulates primary sensory neurons (Someya et al., 2003).

Histamine formation was delayed by salting, regardless of brine concentration, during storage of mackerel muscle at 5°C and during storage at 25°C; the inhibition effect of histamine was directly related to the increase in brine concentration. It was reported that histamine, tyramine and tryptamine production by *streptococcus cremoris* has been suppressed by the addition of 0.5% of NaCl to the base medium (Babu et al., 1986). Studies show that presence of sodium chloride increases tyrosine decarboxylase activity and reduce histidine decarboxylase activity. At the content of sodium chloride 3.5 % the ability of *Lactobacillus buchneri* to form histamine is partly inhibited and at the content of 5.0 % its formation is stopped. Sodium chloride in concentrations above 1-2% reduces the growth rate and delay histamine formation by most Gram-negative histamine producing bacteria (Okuzumi et al., 1984a; Yamanaka et al., 1985; Ramesh and Venugopal 1986; Yamamoto et al., 1991; Ababouch et al., 1991; Wendakoon and Sakaguchi 1993; Morii et al., 1994; Aytac et al., 2000). Glycine (10% w/v), sorbic acid (0.l-0.2% w/v) and 10% (w/v) of citric, malic and succinic acids have a diminishing effect on biogenic amine synthesis (Kang and Park, 1984a; Kang and Park, 1984b).

Sodium hexametaphosphate and sodium sorbate has been shown to reduce histamine production in foods (Kang and Park 1984; Shalaby and Rahman 1995; Shalaby 1996). Succinic acid, citric acid, malic acid, and D-sorbitol inhibited decarboxylase activity and the resulting histamine production in mackerel was reduced when stored for 10 d at 25 °C (Shalaby 1996). Citric acid added in 1% in pickled cabbage shows slight decrease in biogenic amines at a salt level of 6, 8, or 10% (Yuecel and Ueren 2008). Shalini et al., (2001) reported that potassium sorbate has extended the shelf

life of seafood. Potassium sorbate, Sodium nitrites and ascorbic acid added to the sausage showed a significant inhibition of biogenic amine accumulation (Bozkurt and Erkmen 2004, Kurt and Zorba 2009). This confirms the results of Bozkurt and Erkmen (2004). Incorporation of 0 to 1% glucono-delta-lactone into meat reduced histamine and putrescine production through a pH decrease in meat (Maijala et al 1993). The addition of sugar may also slightly decrease biogenic amine formation (Bover-Cid and others 2001a). Glycine was applied to Myeolchi-jeot, the overall production of biogenic amines was reduced by 63 to 73%. Biogenic amines in other fermented fish products may be decreased using glycine as a food additive (Mah and Hwang 2009a). The authors concluded that food additives inhibit the amine forming activity of microorganisms.

Some of the authors have pointed their potential harmful effects of preservatives on biogenic amine accumulation, For example, the addition of preservatives has been reported to increase biogenic amine formation during sausage production (Komprda and others 2004). Recently, it was reported that curcumin in turmeric inhibits diamine oxidase (Bhutani and others 2009), which may inhibit biogenic amine decline. When sodium sorbate and sodium hexametaphosphate were applied to sardines as a preservative, a putrefactive odor was developed within 2 d at chill storage (Kang and Park 1984). Histamine levels recorded in the presence of 3.50% salt or 0.02% sodium nitrite were very similar to those found in the additive-free control broth (Teodorovic et al., 1994). Moreover, proteolysis is observed during ripening of salted anchovies, resulting in the release of peptides and free amino acids including histidine (Hernandez-Herrero et al., 2002). Presence of salted fish with histamine formation is maybe due to the presence of halophilic or halotolerant microorganisms. For instance, Hernandez-Herrero et al., (1999) found that *Staphylococcus epidermidis*

and *Staphylococcus capitis,* isolated from salted anchovies, showed a powerful histamine-producing activity. When glucose and sucrose were added to mackerel muscle it was observed more histamine formation than in ground muscle. Other disadvantages of preservatives use are a lack of existing reports on their effectiveness against biogenic amines in foods and the lack of end user acceptance (Bjornsdottir, 2009).

In conclusion food additives and preservatives that needs further study into the effectiveness in inhibiting biogenic amine production in food and find out their positive effect on delaying biogenic amine formation in variety of food systems

Microorganisms involved in the biogenic amine production

Most of the microorganisms have the capacity to produce biogenic amine, including gram positive and gram negative bacteria of different genera and species, such as *Bacillus, Salmonella, Citrobacter, Clostridium, Escherichia Klebsiella, Proteus, Shigella,, Photobacterium, Pseudomonas* and the *lactic bacteria Lactobacillus, Streptococcus and Pediococcus*are able to decarboxylating one or more amino acid (Brink et al., 1990; Huis in't Veld et al., 1990; Kim et al., 2003; Chen et al., 2010), although such capacity is generally depend on a strain-level characteristics of such organism (Gardini et al., 2006). Biogenic amine production by bacteria was based on the availability amino acids in food and decarboxylases synthesizing capacity of organism (EFSA, 2011). Histamine is produced in fish by microorganisms capable of producing the enzyme histidine decarboxylase (HDC). The histidine decarboxylases produced by bacteria catalyze the conversion of free histidine, naturally present at high levels in the muscle of some fish, to histamine. Gram-negative and Gram-positive bacteria can both produce

histidine decarboxylase but the forms of the enzymes differ (EFSA, 2011; Bjornsdottir-Butler et al., 2010). The gram-positive bacteria produce heterometric histidine decarboxylase enzyme that contains pyruvoyl group (Konagaya et al., 2002) whereas the histidine decarboxylase of animals and gram- negative bacteria are contain pyridoxal 5-phosphate (Kamath et al., 1991). Products such as fermented fish sauce could contain histamine from each of the two types of decarboxylase, first from gram-negative bacteria that already present in the fish earlier to sauce production and also histamine from the Gram-positive form of histidine decarboxylase formed during the fermentation step. In the same way, other biogenic amines (putrescine, cadaverine, and tyramine) are synthesized by decarboxylases produced by Gram-positive and Gram-negative bacteria.

Reports found that *Morganella psychrotolerans*, low temperature-adapted bacteria, and a strong histamine-producer could play a role in scombroid poisoning (Emborg et al., 2006; Kanki et al. 2004). Biogenic amine producing bacterial species and strains vary considerably in amounts of amine formation, and the kind of spoilage bacteria present in the fish. For eg. *M. morganii*, *P. vulgaris* , and *K. pneumoniae* are capable of producing histamine>1000mg/kg in the culture broth (Lopez-Sabater et al., 1996; Rawles et al., 1996; Kim et al., 2001) and they have rarely been detected in fresh fish, but have mostly been isolated from fish spoiled under prescribed storage environment (Ababouch et al., 1991; Kim et al., 2001). The bacterial species such as *Pseudomonas spp., Photobacterium spp., Aeromonas spp. and Vibrio alginolyticus,* are weak histamine producer, forming <500mg/kg in the culture broth and are occur naturally in marine environment (Frank et al., 1985, Middlebrooks et al., 1988; Morli et al., 1988). Biogenic amine content in raw fish is linked to the type of natural microbial flora associated with fish, while

in processed fish due to cross-contamination during handling processing and storage (Kim et al 2003; Allen et al., 2005). Therefore, food contact surfaces should be sanitized regularly during fish processing to avoid introducing biogenic amine producing bacteria from contaminated surfaces to fish.

Other factors

Amino acids present in the food play an important role in the production of biogenic amines in foods. Free amino acids present in the food depends on either occur as such in foods, or may be liberated through proteolysis. Microorganisms with high protein hydrolysis enzyme activity potentially enhance the availability of free amino acids results the risk for biogenic amine formation in food systems. Elimination of the α-carboxyl group from an amino acid leads to the corresponding biogenic amine. The names of several biogenic amines correspond to the names of their originating amino acids: tyramine from tyrosine, tryptamine from tryptophane, histamine from histidine, β-phenylethylamine from phenylethylalanineetc (Beutling, 1996). In plants and some microorganisms, another pathway exists to form putrescine from arginine through agmatine. Lysine is decarboxylated with lysine decarboxylase to produce cadaverine, although it can also be produced by ornitine decarboxylase if the concentration of ornitine is low, but that of lysine is high (Lovaas, 1991).

Oxygen supply also appears to have a significant role on the biosynthesis of amines. *Enterobacter cloacae* produces about double the quantity of putrescine in aerobic condition compared with anaerobic conditions, and *Klebsiella pneumoniae* synthesizes significantly low cadaverine but acquires the ability to produce putrescine under anaerobic conditions (Halsz et al., 1994). On the other hand, it has been found that

reducing the redox potential of the medium also stimulates biogenic amine production (Arnold and Brown, 1978; Hal&z et al., 1994). Histidine decarboxylase activity of *Proteus morganii* is reduced in atmospheres of 80% CO_2 (Watts and Brown, 1982). Modified atmosphere packaging gives slight decrease of histamine producing bacteria in big-eye tuna and the histamine formation was not attributable to the histamine-producing bacteria studied *Morganella morganii*, *Klebsiella pneumoniae* and *Hufniaalcei* (Oka et al., 1993). Suppression of histidine-decarboxylase has also been observed when the quantity of histamine is accumulated in the medium (Omure et al., 1978). The presence of histamine had reduced the effect on the histidine decarboxylation activity of *Photobacterium histaminum* C-8; histamine, agmatine and putrescine reduced the histidine decarboxylation activity of *Photobacterium phosphoreum N-14* (Kurihara et al., 1993).

A thorough understanding of the mechanisms by which biogenic amines are being formed is necessary to stop their formation. Generally, biogenic amines production in food can be controlled by strict use of good hygiene and sanitary practices in both raw material and processing environments with corresponding inhibition of spoiling microorganisms. In summary the concentration of biogenic amines in fish products will depend on the species and composition of fish, the way the fish is handled, time, conditions, and temperature of storage of the fish. This combination of different factors can lead to highly variable levels of contamination within lots of fish, and even within individual fish, and has implications for the efficacy of testing schemes to assess the safety of fish and fish products with respect to biogenic amine contamination.

2.1.6 Effects of various foods processing on biogenic amines formation

Biogenic amine accumulation in food can be controlled by preventing microbial growth (Jimenez-Colmenero, 2004) or by inhibiting the decarboxylase activity of microbes (Wendakoon and Sakaguchi 1995). The prevention of biogenic amine formation in food has achieved by using high-quality raw material (Park et al., 2010), good manufacturing practice (GMP) (Emborg and Dalgaard, 2007), using appropriate storage conditions (Carelli, 2007), using temperature control (Emborg and Dalgaard, 2008a), the use of amine-negative starter culture (not able to decarboxylate amino acid into biogenic amines) or biogenic amine oxidizing cultures (biogenic amines oxidized into aldehyde, hydroden peroxide, and ammonia) for fermentation (Bover-Cid and others 2000a; Dapkevicius and others 2000; Suzzi and Gardini 2003; Nieto-Arribas and others 2009), the use of microbial modeling to develop favorable surroundings to delay biogenic amine formation (Neumeyer and others 1997; Emborg and Dalgaard 2008a, 2008b; Dalgaard, 2009), packaging techniques (modified atmosphere or vacuum packaging) (Mohan and others 2009), High hydrostatic pressure (HHP) (Bolton and others 2009), irradiation (Naila et al., 2010; Kim and others 2003), and food additives (Naila et al., 2010; Mah and Hwang 2009a). Well-organized approaches to prevent biogenic amine production involve the combined effect of an existing method and emerging methods, such as the combination of temperature control, High hydrostatic pressure and amine-negative starters (LatorreMoratalla and others 2007). However, optimization of such an approach is required. Once biogenic amines are produced, it is very difficult to destroy them by processing. Therefore, biogenic amine formation by micro organisms should be controlled by strict use of good sanitation and hygiene practices in both raw material and

manufacturing environment, with corresponding inhibition of spoiling microorganisms (Silla Santos, 1996).

2.1.7 Toxicological Aspects of Biogenic amines

While endogenous concentrations of histamine, tyramine and putrescine are necessary and are required for normal physiological function in man and animals, they are toxic when large doses enter the circulatory system. This often results in poisoning symptoms, which affect a wide range of organs (Taylor, 1986).

The most common and notorious foodborne intoxications caused by biogenic amines are associated with histamine. Several outbreaks of histamine poisoning have reported after eating cheese or fish. However, exact statistics about its occurrence do not exist because poisoning incidents are often unregistered due to mild symptoms, lack of sufficient reporting systems, or misdiagnoses by medical personnel of histamine poisoning as a food borne infections (FAO, 2004). An ingestion of 5-10 mg of histamine can be considered as toxic to some sensitive people. Whereas for common people 10 mg is considered as allowable limit for safe consumption, 100 mg may cause slight, intermediate poisoning and 1000 mg is potentially dangerous (Ascar, 1986, Lehane, 2000). Consumption of 100-800mg /kg tyramine and 30 mg / kg phenylethylamine have been reported as toxic level (Ten Brink et al., 1990).

Histamine poisoning results dilatation of peripheral blood vessels, mainly arteries, results in hypotension, flushing, and headache. Histamine delivers its toxic effects by binding to receptors such as H1, H2, and H3 on cellular membranes in the gastrointestinal, cardiovascular respiratory and immunological systems and the skin (Bentley, 1995). Histamine assisted with H1 receptors resulting tightening of intestinal smooth muscle, abdominal

cramps, diarrhoea, and vomiting. Histamine bind with both H1 and H2 receptors promotes increased capillary permeability, resulting in symptoms such as edema, urticaria, hemoconcentration, and increased blood viscosity (Owen et al., 1980). Gastric acid discharge is reduced by histamine with H2 receptors located on the parietal cells (Stratton, 1991). Itching and Pain associated with the urticarial lesion may be due to sensory and motor neuron stimulation through H1 receptors (Bentley, 1995).

Histamine exhibits a direct stimulatory action on the heart and speed up heart contractility. Contraction is mediated by H1 receptors, while relaxation is associated with H2 receptors (Shahid et al., 2009).In individual, the main action of histamine on vascular smooth muscles is contraction. This contraction is mainly seen in the bronchi and intestines. In histamine intoxication, the tightening of intestinal smooth muscle is particularly noticeable, as histamine enters the gastrointestinal tract firstly. Contraction of intestinal smooth muscle leads to the abdominal cramps, diarrhoea, and vomiting which are often noted in cases of histamine poisoning (Taylor, 1986). Histamine is also a strong stimulant of both sensory and motor neurons. This stimulation may be important in producing the itching and pain that frequently accompany the urticarial lesions in histamine poisoning. This neural stimulation is mediated by H1 receptors (Nuutinen and Panula, 2010).

Cadaverine and putrescine are considered histamine potentiators, which may explain the lack of toxicity of pure histamine in human oral challenge studies. Study in guinea pigs shows, putrescine and cadaverine improved the histamine-related mortality (Bjeldanes et al., 1978; Vasseur et al., 1968). As data of their potentiating effects, cadaverine and putrescine were confirmed to be functional inhibitors of DAO and HMT in a rat jejunal model (Taylor and Lieber, 1979, Bjeldanes et al., 1978; Shalaby, 1996). In an *in vivo* study carried

out in rats, both putrescine and cadaverine increased the amount of un-metabolized histamine, but decreased the amount of its metabolites in urine (Hui and Taylor, 1985). The lowest level of cadaverine or putrescine that potentiates histamine toxicity is unknown. On the other hand, it must be taken into care that secondary amines such as putrescine and cadaverine can react with nitrite to produce heterocyclic carcinogenic compound such as nitrosamines, nitrosopiperidine and nitrosopyrrolidine (Huis in't Veld et al., 1990).

In human being, tyramine performs as a catecholamine (including norepinephrine, dopamine, epinephrine) discharging agent, resulting in increased blood pressure. While tyramine is physiologically metabolized by monoamine oxidase (MAO), a hypertensive crisis can result when a person who takes MAO inhibitor (MAOI) drugs and also consumes foods with high histamine content. This state, also called the tyramine pressor response, is characterized by an increase in systolic blood pressure of 30 mmHg or more. Transfer of norepinephrine from neuronal storage vesicles by increased tyramine intake is thought to cause the vasoconstriction, increased blood pressure and heart rate. In addition to the hypertensive effect, dietary tyramine intake has also been related with migraine headaches in select populations, and the mechanism has been linked to tyramine as a neurotransmitter (Jansen et al., 2003).

In animals, tyramine has a low acute oral toxicity of more than 2000 mg/kg body weight. It causes a dose-dependent increase in blood pressure. When using a MAOI, the intake of approximately 10 to 25 mg of tyramine is essential for a severe reaction compared to 6 to 10 mg for a mild reaction. For adults, levels of 100-800 mg/kg of dietary tyramine have been suggested as acceptable, and levels >1080 mg/kg as toxic (Tenbrink et al., 1990). In individuals using MAOI drugs, ingestion of 60 mg/kg of tyramine can cause

migraine headaches, while 100-250 mg/kg will produce a hypertensive crisis (Silla Santos, 1996).

Scombrotoxin Fish Poisoning (SFP)

SFP is a world-wide food safety problem and is a common cause of fish poisoning that occurs in humans. The food poisoning is caused by heat-stable scombrotoxins, presumably arising from bacterial action in fish. Though detailed components of scombrotoxins have not been identified, it is generally accepted that biogenic amines, especially histamine, play an important role in the pathogenesis of SFP. The incriminated fish usually contain abnormally high levels of histamine due to bacterial activity resulting from inappropriate handling, processing or storage conditions, and histamine has been implicated, at least in part, as an important causative agent. So, SFP is also called histamine fish poisoning (HFP). While SFP shares some symptoms with histamine intolerance and histamine induced adverse effects, there are differences. In contrasting to histamine intolerance and histamine induced effects, Scombrotoxin Fish Poisoning may involve the occurrence of other toxic decomposition products or components exclusive to fish (Hungerford, 2010). In addition, contrasting histamine intolerance SFP occurs not only in susceptible individuals, but also those with normal capacity for histamine degradation.

Symptoms

A range of symptoms of SFP have been seen among humans. Poisoned individuals may show one or more of these symptoms, and the harshness of response to the contaminated fish may vary. In several reports, exacerbation of asthma and more serious cardiac symptoms were reported (Ascione et al., 1997; D'Aloia et al., 2011). The symptoms typically develop quickly (from 5

minutes to 2 hours after ingestion of spoiled fish, with a usual duration of 8-12 hours and with symptoms normally no longer observed after 24 hours. Although symptoms may continue up to several days, there are no known long-term sequelae. SFP is considered to be rarely if ever fatal. According to report from the United States Centers for Disease Control and Prevention (CDC) for the period from 1998-2002, there were 463 cases reported and no deaths (CDC, 2006). According to the data from the Japanese Ministry of Health, Labour and Welfare for the period from 1998-2008, there were 89 incidents, 1577 cases reported and no deaths (Toda et al., 2009).

Diagnosis

The finding of Scombrotoxin Fish Poisoning is mainly dependent on the symptoms, history of food allergy, and the consumption of contaminated fish. The diagnosis can be confirmed by detecting high levels of histamine in the concerned food, meal leftovers or a similar product collected from the same source (Ferran and Yebenes, 2006; Predy et al., 2003).

Table: 2.1 Common symptoms of scombrotoxin fish poisoning

Type	Symptoms
Cardiovascular	Flushing, rash (urticaria), hypotension, headache, tachycardia
Gastrointestinal	Abdominal cramps, diarrhoea, vomiting
Neurological	Pain, itching
Other	Oral burning sensation, peppery taste, nausea, swelling of tongue

Treatment

Antihistamine treatment is the best method of therapy for SFP. Symptoms usually reduce rapidly after such treatment. Both H1 (e.g., diphenhydramine) and H2 antagonists (e.g., cimetidine) have been used for the treatment of histamine poisoning. Since the adverse responses are self-limiting and will resolve with in short time, pharmacological involvement may not be

necessary in mild cases and only need maintenance support (e.g., fluid replacement) (Taylor, 1986).

2.1.8 Relationship of Biogenic Amines to other Quality and Spoilage Indices

Several attempts have been made to find the relationships between biogenic amines and the most widely used indices of freshness or spoilage (Sims et al., 1992; Silla Santos, 1996). Biogenic amines, especially histamine, putrescine and cadaverine have been recommended as indicators of spoilage of some foods, such as fresh fish, milk, meat, vegetables and fermented foods (Bozkurt, et al, 2004). Among the biogenic amines, histamine has been the most studied in relation to sensory changes. Ababouch et al. (1996) found that sardine stored at 0 and 20°C was rejected for consumption due to high histamine levels before being rejected by the sensory panelists. However, Guizani et al. (2005) reported that tuna stored at ambient temperature spoiled and became unsafe for consumption within one day but those stored at 0°C spoiled after 12 days. In this study histamine did not exceed 5 mg/100 g fish FDA safety level during 17 days of ice storage. Yamanaka (1987) proposed agmatine can be used as a freshness indicator in common squid (*Tobarodespacificus*). Agmatine was detected in small amounts in the fresh muscle and the concentration exceeds to 30 mg/100g at the stage of initial decomposition and reached the level of 40 mg/100g at the stage of advanced decomposition. Mietz and Karmas (1977) found that Biogenic Amines Index (BAI) can estimate the quality of rockfish, lobster shrimp, and salmon. Dawood et al. (1988) point out that putrescine and cadaverine could be used to evaluate the freshness of rainbow trout (*Salmon irrideus*). Okozumi et al. (1990) indicated that increased levels of putrescine and cadaverine were identified at the spoilage stage of horse mackerel meat when Pseudomonas

spp. was the dominant bacterial flora. Sims et al., (1992) found a good correlation between sensory quality and amount of cadaverine and putrescine in skipjack tuna. Veciana-Nogues et al. (1997) proposed a biogenic amine index calculated from the sum of histamine, putrescine, cadaverine, and tyramine for tuna quality evaluation.

Many studies have attempted to find a relationship between levels of TMA and biogenic amines in order to enable prediction of fish safety from its TMA content. However, this relationship seems to be strongly affected by the concentration of free amino acids in fish. Trimethylamine (TMA) is one of the main volatile amines produced by spoilage bacteria during fish spoilage (Laycock and Regier, 1971; Gram et al., 1987; Gram and Dalgaard, 2002). Veciana- Nogues et al., 1990) reported the relationship between biogenic amines and fish spoilage indicators. The study found a high correlation coefficient between changes in histamine and TMA during the storage of anchovies with high histidine content at both refrigeration and room temperatures. However, this relationship did not exist in low-histidine fish such as emperor and barracuda (Shakila et al., 2003). The effect of fish histidine content on the relationship between histamine and TMA is illustrated by the findings of Baixas-Nogueras et al. (2001) in hake where TMA increased to a level at which the fish was rejected after 10 h of storage at 18-20°C or 21 h at 8-10°C but histamine was not detected at these times.

The absence of changes in histamine levels in some studies where fish were stored at low temperature (Guizani et al., 2005) could be explained by the presence of histamine-decomposing bacteria which have the potential to hydrolyze histamine at low temperature. Many psychrotrophs such as Pseudomonas species have the ability to decompose histamine at low temperature (Sato et al., 1995). At higher storage temperatures (30°C) no

reduction in histamine content was found (Sato et al., 1994). This observation was explained by domination of histamineproducing bacteria (Sato et al., 1994). Sato et al. (1995) found that *Pseudomonas putida* played a major role in histamine decomposition at low temperature since it dominated 80–100% of the histamine-decomposing bacteria when the histamine content decreased and disappeared. Earlier histamine development in fish stored at ambient temperature (Ababouch et al., 1996) could be explained by the presence of the main histamine producers, such as *Morganella morganii* and *Proteus vulgaris*, which produce their highest histamine level at 25°C (Kim et al., 2001a).

2.1.9 Analytical methods for the determination of biogenic amine

For the determination of biogenic amine in proteinaceous foods number of analytical methods were developed involves extraction of the biogenic amines from the food matrix and the determination of the biogenic amines by using analytical methods (Silla-Santos, 1996).

The complex food matrix, the presence of potentially interfering chemical compounds, and the occurrence of several biogenic amines simultaneously are typical troubles encountered in the analysis (Arlorio et al.,1999).

Extraction or pre-clean up with suitable extracting reagent is the most critical step in the detection procedure of biogenic amine(Silla-Santos, 1996). The extraction of biogenic amine from a solid matrix is usually carried out with acids such as 0.1M hydrochloric acid (HCl), 0.6m perchloric acid ($HClO_4$), ($C_2HCl_3O_2$) methanesulfonic acid (CH_4O_3S) or with 5-10% trichloroacetic acid. Organic solvents such as methanol at 60^0C, butanol, butanol-chloroform at basic pH, acetone, acetonitrile-$HClO_4$ or dichloromethane- $HC1O_4$ can also be used. The extraction efficiencies of these solvents is influenced by many factors such as nature of the biogenic amine,

type of food, type of acid, type of organic solvent, salt used for saturation, pH, time and type of stirring etc. The solid-phase extraction (SPE) has provided a more efficient choice than classical liquid-liquid extraction because of the availability of high quality sorbent materials and destroying property of organic solvents is avoided.

The number and variety of analytical methods developed for the separation and quantification of biogenic amine levels in many foods (Hungerford, 2010), including the well-established AOAC fluorometric method (AOAC 977.13), the spectrofluorometric method, ELISA methods, the colorimetric enzyme test (Sato et al., 2002), and several chromatographic methods that can measure different biogenic amines (Duflos et al., 1999; Veciana-Nogues et al., 1997). Chromatographic methods includes ranging from simple, most popular and inexpensive thin layer chromatography (TLC) procedures to more powerful, and rapid liquid chromatography-mass spectrometry (LC-MSMS) methods (Hungerford, 2010). TLC is does not require any special equipment, but most available methods shows too much time needed for analysis and/or semi quantitative or inaccuracy results. HPLC with pre-column derivatization using benzoyl chloride, dansyl and dabsyl chloride, 9-fluorenylmethyl chloroformate, fluoresceine. (Mietz and Karmas, 1978; Hui and Taylor, 1983; Malle et al., 1996) or post-column derivatization with o-phthalaldehyde and ninhydrine (Gloria et al., 1999; Brillantes and Samosorn,2001) are the frequently reported technique for biogenic amine separation and quantification (BOMKE et al., 2009). Most of the high performance liquid chromatography (HPLC) uses reversed-phase technique for histamine separation in fish. Other most common separation-based methods include TLC (Bajc and Gacnik, 2009), ion chromatography (Cinquina et al., 2004a), paper electrophoresis (Sato et al., 2002, 2006),

capillary electrophoresis (Zhang and Sun, 2004), and GC-MS (gas chromatography mass spectrometry) (Marks and Anderson, 2006). The most high speed method for detecting histamine is based on flow injection analysis (FIA) and is able to screening 60 samples extracts/hour (Hungerford et al., 1990). Enzymatic methods with radio immune assays and ELISA (enzyme-linked immune sorbent assay) system have been used to analyze histamine (Stratton et al., 1991). Enzymatic methods are important for their selectivity and flow injection has been applied in combination with enzyme electrodes for easy automation (Watanabe et al., 2007). Several commercial test kits are developed, based on selective antibodies reaction (Lehane and Olley, 2000; Kose et al., 2009). Commercially available rapid test kits based on immunoassay methods for histamine analysis became well accepted because of their user friendliness and low time requirements compared to those of usual analytical techniques. Recently many authors (Kose et al., 2011; Tahmouzi et al., 2011; Tao et al., 2011; Hungerford and Wu, 2012) published different methods for rapid determination of biogenic amine in fish.

2.1.10 Limits of histamine level in fish and fishery products

As per the FDA regulation (FDA, 2011), the toxicity and defect action levels of histamine, established for tuna, mahi-mahi, and related fish, are the 50mg/100g and 5mg/100g, respectively. The term "defect action level" defines to the level of histamine naturally or inevitably occurring in foods without showing a significant hazard for humans. According to the EU Regulation No 2073/2005 nine samples should be taken from each batch of fish species of the following families: *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae*. These samples must fulfill the following requirements:

- Mean value of all samples must not greater 10mg/100g;
- Two samples may be >10mg/100 but <20mg/100g;
- No sample may exceed 20mg/100g.

However, fish from these families that have undergone enzymatic changes in salt may have high amount of histamine, but not more than twice the above values.

According to Commission Regulation EC No 2073/2005, a limit of 200 mg/ kg fresh fish for histamine has been established in fish species associated with a high amount of histidine. Almost similar regulation exists in regulation pertaining to other countries. The latest regulation, however, prescribes higher values equal to 400 mg/kg for products like fish sauce as they being a liquid fishery product, histamine can be expected to be evenly distributed. Also, European Food Safety Authority conducted limited studies points out that a concentration of 50mg histamine is considered as no observed adverse effect level (NOAEL), the maximum concentration of histamine that would not cause an adverse effect would be equal to 200 mg/kg (FAO/WHO, 2012).

Table: 2.2 FSSAI Limits of histamine applicable to fish Species with high amount of free histidine

Product Category	Histamine Level
Raw/Chilled/Frozen Finfish	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Dried/ Salted and Dried fishery products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Thermally Processed Fishery Products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Smoked fishery products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Fish Mince/Surimi and analogues	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Battered and breaded fishery products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Other Ready to Eat fishery products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Other value added fishery products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Other fish based products	n=9, c=2; m=100 mg/kg, M=200 mg/kg
Fermented Fishery products	n=9, c=2; m=200 mg/kg, M=400 mg/kg
Fish Pickle	n=9, c=2; m=200 mg/kg, M=400 mg/kg

Where:n: Number of units comprising the sample c: Maximum allowable number of defective sample units, m: Acceptable level in a sample, M: Specified level when exceeded in one or more samples would cause the lot to be rejected

The risk of histamine poisoning could be effectively controlled by following the principles of good manufacturing practices and Hazard Analysis Critical Control Point (HACCP) system appropriate to fish and fishery products. Maintaining the commodity under controlled temperature regime by appropriate icing practices, hygiene handling and transportation under controlled conditions are to be strictly adhered to in order to avoid the onset of histamine issues.

2.1.11 Reference

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Chapter

3

COMPOSITION OF SQUID MUSCLE WITH SPECIAL EMPHASIS ON AMINOACIDS

Contents

3.1 Introduction

The consumption of cephalopods especially squid has increased recently mainly as chilled and frozen ready meals (Barbosa &Vaz- Pires, 2004). Squids are popular worldwide, because of their rich nutrition, subtle flavor and chewy texture (Deng et al., 2011) and offer a good market price (Okuzumi and Fujii, 2000). Squid and cuttle fish contributed to one of the major export value item accounting for a share of 10.24 % of the total US $ earnings during the period 2013-14. The percentage of the edible portion of the cephalopods is remarkably high varying from 60 to 80% of the total weight depending on species, size, and sexual maturity, whereas in fish the percentages is only 40-70% depending on the fish. (Lee, 1994; Sikorski & Kolodziejska, 1986). Squid contain high percentage of protein and all the essential amino acids especially lysine which is essential for growth (Torrinha et al., 2014). In addition to that it contains appreciable amount of fatty acids,

cholesterol, vitamin E and minerals (Mathew, 1999). The nutritional composition of squid has been reported (Joseph et al., 1977; Okuzumi and Fujii, 2000; Forsythe et al., 2002; Thanonkaew et al., 2006; Pierce et al., 2008; Okeyo, et al., 2009; Chakraborty et al., 2016). However, the composition could vary among species due to geographical differences of fishing grounds, feed intake, migratory swimming and sexual changes in connection with spawning. Besides, biochemical profile can vary with body part. Therefore, the present study was designed to evaluate the chemical properties and nutritional composition of the edible part of Indian squid (*Loligo duvauceli*) and the information will be useful in finding optimum processing and storage conditions in order to preserve the quality of squid.

3.2 Review of Literature

Considering the promising perspective for the utilization of squid, studies on the chemical composition of squid is very important (Baldwin, 1933). Proximate composition typically comprises water, protein, fat, and ash, expressed as percentage of the weight and are group specific and species-specific. Squid is rich in macronutrients and micronutrients such as proteins, lipids, amino acids, vitamins which are essential for healthy human life (Dileep et al., 2012; Bano et al., 1992). There are various reports are available on the biochemical compositions cephalopods (Sreeja, et al., 2012; Tze-kuei-chiou et al., 2000; Lakshmanan and Balachandran 2000; Ozyurt et al., 2006). Pandit and Magar (1972) have analyzed the proximate composition of *Loligo vulgaris* and reported that the species contains 79.73 % moisture, 0.81% fat, 16.52% protein and 1.49% ash. Shchenikova et al., (1987) reported on the relatively low lipid content in cephalopods (0.5-2.6%). Squids are rich in essential fatty acids like eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) and having antithrombotic, antiatherogenic and anti-inflammatory

properties (Wall et al., 2006). Chu et al., (1992) observed higher myofibrillar proteins and NPN and lower moisture content in squid mantle than octopus. Nagaraj (1994) reported relatively higher protein and fat content in squid (*Loligo duvauceli*), while Moustafa et al., (1998) noticed small changes in the proximate composition of the frozen stored and hot smoked squid (*Loligo vulgaris*) mantles and tentacles for 6 months and the products were found good for consumption throughout the storage period. According to Lakshmanan and Balachandran (2000), proximate composition of the cephalopod flesh shows moisture 75-80%, crude protein 16-21 %, crude fat 1.0-1.5% and ash 1-2%. Edible portion in squid is about 76% and that in cuttlefish is about 65%, with a higher value of protein (18-20%) than many fishes and the protein is composed of 20 total amino acids of which belong to a group of essential amino acids. (Lakshmanan and Balachandran, 2000). Because of high nutritive value and better taste, cephalopods are widely accepted as a choice food in various parts of the world (Takasashi, 1974; Roper et. al, 1984 and Sarvaiya, 1990).

3.3 Materials and Methods

3.3.1 Raw material

The Indian squid (*Loligo duvauceli*) was collected from the fish landing centre at Fort Cochin, India and were transported to the laboratory in iced condition. Raw material had an average length of 55±14 cm and weight 345±14 g. The squid samples were iced (flake ice) in ratio of 1:3 in an insulated boxes and were placed in a thermo-statically controlled chill room (0 ± 2^0C) until unacceptable. Edible portion (squid mantle & head) was separated and used for the analysis in triplicate. The day the squid were captured was designated as 0th day for the purpose of this study and the specimens were sampled every

2nd day for various analyses like moisture content, crude protein, crude fat, and ash content.

All reagents and solvents used in this study were of analytical grade. Standards of fatty acid methyl esters, amino acids were purchased from Sigma Aldrich (Steinheim, Germany).

3.3.2 Determination of moisture

Moisture content of the sample was determined by oven drying method according to the AOAC (2000). The moisture content was determined by 10gms of the sample was dried at 105°C in thermostatically controlled hot air oven. The samples were taken in pre-weighed glass dish with cover and kept in oven till the weight become constant. The weight was checked for constant weight by repeatedly heating and then cooling the sample in a desiccator. The percentage solid was determined from the above experiment by using the formula.

$$\text{Percentage solid} = \frac{\text{Weight of drysample}}{\text{Weight of wet sample}} \text{x}100$$

The percentage moisture was calculated by subtracting solid weight % from l00.

3.3.3 Determination of Protein

1gm of homogenized sample was used for determining the crude protein content using Micro Kjeldahl method (AOAC 2000). Sample was accurately weighed into a digestion tube. About 2 gms of digestion mixture ($CUS0_4$ and K_2S0_4 as a catalyst in the ratio 1:8) and 10 ml of concentrated H_2S0_4 were added to the sample taken in a digestion tube. The samples were digested to a clear solution in a digestion unit. 50 ml of distilled water was added to the cooled tube slowly till no heat was generated on adding water.

The solution was made up to 100 ml. Pipetted out 5 ml of the prepared sample into Kjeldahl micro distillation apparatus. The bottom end of the condenser was fitted to a delivery tube, which was immersed in 10 ml of 2% boric acid solution with added Tachiro's indicator. 40% NaOH was added to the sample in the distillation unit to make it alkaline. The ammonia thus produced on steam distillation was absorbed into the boric acid solution. The distillate collected was back titrated against standard H_2S0_4 using Tachiro's indicator and determined the nitrogen content. The nitrogen content thus obtained was multiplied by a factor 6.25 to obtain the crude protein content of the sample.

$$\% \text{ Protein} = \frac{\text{V x0.14x6.25x100}}{\text{1000xV1XW}}$$

Where,

V = Total volume of the digest

V1 =Volume of digest for distillation

W = Weight of sample for digestion

3.3.4 Determination of Crude fat

Fat content of the moisture free sample was determined by extracting the fat by using a suitable solvent by soxhlet extraction method (AOAC 2000). About 2 grams of the sample was accurately weighed into an extraction thimble, and was placed in the extractor. The extractor was connected to a pre-weighed dry receiving flask and a water condenser. Petroleum ether (B.P.40-60°C) was used as the solvent. The unit was heated over a water bath and the temperature was controlled at 40°C- 60°C so that the solvent boiled continuously and siphoned 5 to 6 times per hour. Extraction was continued till the solvent in the extractor became colourless and fat free. The solvent in the receiving flask was evaporated completely and weighed for fat content.

$$\% \text{ Fat} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \text{x}100$$

3.3.5 Determination of Ash

Ash content was determined by the incineration of the sample (AOAC 2000). 2 grams of sample was taken in a pre-weighed silica crucible and the sample was charred on low heat. Then it was then kept at 550°C in a muffle furnace to get a white ash, which was cooled in a decicator and weighed.

$$\% \text{ Ash} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \text{x}100$$

3.3.6 Determination of Lipid Profile

The total lipid content of the tissues was estimated by the method of Folch et al (1957). The determination of fatty acid profile of lipid extracted from squid was carried out in Thermo Trace Gas chromatograph equipped with flame ionization detector and Varian FFAP column (25m 0.32mm 0.3µm #CP 7485) using N_2 as carrier gas. In GC, oven temperature was initially held at 110 ^{0}C for 4 min and then set to increase up to 240 ^{0}C at a rate of 2.7 ^{0}C min-1, held at 240 ^{0}C for 5 min. Fatty acids separated were identified by the composition of retention times those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried out by Thermochrom card software. Individual fatty acids were expressed as a percentage of total fatty acids.

3.3.7 Determination of Mineral

The minerals were analyzed by dissolving the ash in dil.HCl (6 N) and estimated using atomic absorption spectrophotometer (*AAS VARIAN*, Spectra AA 220), with deuterium background correction, acytelene and air supplied in constant ratio for flame and hollow cathode lamp. The wavelengths (nm) of

light used for analyzing different minerals are 285.2 for magnesium, 213.9 for zinc, 766.5 for potassium, 328.4 for copper, 279.5 for manganese , 248.3 for iron, 240.7 for cobalt, 670.8 for lithium , 228.8 for cadmium, 217 for lead. The equipment was previously calibrated with standards.

3.3.8 Determination of Amino acid Profile

The amino acid composition of the studied squid species was carried out by using HPLC system with Shodexpak p-4219 No. P 207074 column. The amino acid sample was prepared by overnight digestion of 200 mg squid sample in 6 N HCl in sealed test (Ishida et. al.,1981). Then the sample filtered through what man filter paper and the HCl in sample which was used to digest the sample is washed out by flash evaporator. The evaporated sample was washed thoroughly with distilled water to remove the remaining tinge of acid. Washed three times with distilled water to remove the acid content completely from the sample. After complete evaporation of water content, the sample is made up to 1 ml with Buffer A in a vial and then used for injection in HPLC.

3.4 Result and discussion

3.4.1 Proximate composition

The proximate and nutrient analyses of *Loligo duvauceli* are presented in Table 3.1. The study shows that the fresh edible portion of the squid sample contains more moisture, comparatively high protein, low fat and low ash. Similar finding was observed in the tentacles of European squid (*L. vulgaris*) (Servet et al 2011; Abugoch et al 1999). According to Lee (1994), Cephalopods have 20% more protein, 80% less ash, 50-100% less lipid and 50-100% less carbohydrate when compared to fish. The result showed that the highest protein content (18.5±0.02%) was recorded for fresh squid samples and the least protein content (15.0±0.15 %) was recorded for sample stored for

14 days at 0 ± 2^0C. A significant percent decrease ($p\leq0.05$) was found in total protein content probably due to leaching out of amino acids and water soluble protein during ice storage. It is reported that proteins exposed to oxidizing environments are very susceptible to chemical modification, such as amino acid destruction, peptide scission and formation of protein-lipid complexes that results in decrease in protein content (Xiong, 1997; Zamir, et al., 1998; Saeed& Howell 2002).

Moisture analysis shows that initially on day zero, moisture content was found to be 78.2±0.22% and then it was increased significantly during storage to the value of 81.5±0.08% on 14th day at 0 ± 2^0C. The gradual increase in the moisture content in squid sample stored in ice is due to the absorption ice melt water (Reghunath, 1984). The results are in accordance with Zamir et al (1998) in crab; Bao et al (2007) in Arctic Charr (*Salvelinusalpinus*) and Siddique et al (2011) in Puntius species found an increasing trend in moisture content. The moisture content of different species of cephalopods studied and was reported in the range 75-84% (Joseph et al., 1977; Suyama and Kobayashi, 1980; Selvaraj, 1991; Lakshmanan et al., 1993; Tse-Kuei-Chiou, 2000, and Lakshmanan & Balachandran, 2000).

The result presented in the table 3.1 showed the fat content decreased from initial 1.96±0.5% to 0.74±0.11% after 14 days of storage at 0 ± 2^0C and is related to leaching out during the ice storage. Fat content in the sample was comparatively low and less susceptible to oxidation changes.

Results revealed that the ash content decreased significantly from 0.97±0.0% on day zero to 0.87±0.01% on 14^{th} day of storage at 0 ± 2^0C. These results are in agreement with Beklevik et al., (2005) while working on sea bass fillets; Okoyo et al (2009) on Nile perch and Emire et al., (2009) on Tilapia,

(*Oreochromisniloticus*) reported a decrease in total ash content during its storage. Khan et al (2005) reported a strong correlation between the storage period and ash content ($r=0.819$, $p\leq0.0002$) in blue mussels (*Mytilusedulis*) during storage in ice.

Nurjanah et al (2012) reported that proximate analysis of cuttlefish contained 13.16 -13.51% proteins, 0.7-0.9% ash, and 0.8% fat and1-1.4% carbohydrate. Servet et al (2011) studied the moisture, fat, protein and ash contents of tentacles were 80.72%, 1.44%, 16.16% and 1.63% while the same contents for mantle were 78.54%, 1.37%, 18.52% and 1.45% respectively in European squid. The chemical composition, size, and weight of cephalopods are dependent on growth stage, temperature, salinity, oxygen, light, food, competition, social interaction and sex (Forsythe et al., 2002; Okuzumi and Fujii, 2000)

Table.3.1 Proximate composition of *L.duvauceli* stored in 0 ± 2^0C during 14 days storage period

Days	Protein%	Moisture%	Fat%	Ash%
0	18.5 ± 0.02^a	78.2 ± 0.22^a	1.96 ± 0.05^a	0.97 ± 0.0^a
2	18.3 ± 0.04^a	78.7 ± 0.04^b	1.96 ± 0.03^a	0.97 ± 0.00^b
4	17.8 ± 0.07^b	79.1 ± 0.21^c	1.95 ± 0.01^a	0.96 ± 0.01^c
6	17.2 ± 0.16^c	79.8 ± 0.03^d	1.96 ± 0.00^a	0.96 ± 0.01^c
8	16.7 ± 0.03^d	80.1 ± 0.18^e	1.92 ± 0.01^b	0.95 ± 0.01^d
10	15.6 ± 0.22^e	80.6 ± 0.13^f	1.89 ± 0.01^c	0.89 ± 0.05^e
12	15.3 ± 0.06^f	81.2 ± 0.17^g	0.82 ± 0.07^d	0.89 ± 0.01^f
14	15.0 ± 0.15^f	81.5 ± 0.08^h	0.74 ± 0.11^e	0.87 ± 0.01^g

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly ($P<0.05$)

3.4.2 Amino acids composition

Amino acid composition in *Loligo duvauceli* is shown in Table 3.2. Arginine, lysine and leucine represented 50% of the essential amino acids

(EAA) in squid. Glutamatic acid, aspartic acid and glycine represented 61% of the Non-Essential Amino Acids (NEAA). Arginine, leucine and lysine were among the highest component of essential amino acids in *S. officinalis*, *Loligo vulgaris* and *Octopus vulgaris* (Villanueva et al., 2004), as well as in giant squid *Architeuthis sp* and *Rossiamacrosoma* (Rosa et al., 2005; 2006). The amino acids content of the organisms varies with geographical area, species, age and physiological condition (Capillas et al., 2002). Arginine and proline are the main substrates for amino acid catabolism for energy in the cephalopods (Hochachka and Fields, 1983; Mommsen et al., 1982). The availability of arginine tends to increase during anaerobic work. Arginine phosphate is hydrolyzed and the arginine available is condensed with glucose-derived pyruvate to form octopine, the main anaerobic glycolysis end product that accumulates in adult cephalopods during periods of exercise and stress (Hochachka et al., 1977; Storey and Storey, 1978). In addition, arginine with alanine, glutamic acid, and glycine are free amino acids responsible for the formation of flavour.

Table.3.2 Amino acid composition *Loligo duvauceli*

Essential Amino acids	mg/100g wet weight	Non Essential Amino acids	mg/100g wet weight
HIS	6.58±1.32	Asp	15±1.53
Arg	15.12±1.20	Glu	17.04±0.89
Thr	6.47±.50	Ser	6.14±1.17
Val	6.18±0.28	Gly	8.45±0.50
Met	4.13±0.69	Ala	7.75±0.32
lso	7.21±0.61	Pro	6.17±0.26
Leu	11.39±1.72	Tyr	4.35±0.21
Phe	5.25±1.32	Cys	1.96±1.06
Lys	12.51±1.43	-	-
TEAA	78.11±.1.86	TNEAA	66.07±0.25

Values are expressed as mean ± SD

3.4.3 Fatty acids composition

The fatty acid compositions of *Loligo duvauceli* muscles are shown in Tables 3.3. The fat content in squid was very low and polyunsaturated (PUFA) constituted 67.6 % of the total fatty acids followed by 30.22% saturated fatty acids and only 2% monounsaturated fatty acids. The results of the fatty acid analysis reveal that *Loligo duvauceli*is quite rich in polyunsaturated fatty acids. Navarro and Villanueva (2006) found that cephalopods in their early stages of growth show high requirement for PUFA and they are present 53.62% in mantles and 48.42% in tentacles. Villanueva (2000) also reported that C22:6 n-3 (DHA) were the dominant PUFAs in muscle lipids. DHA and 20:5 n-3(EPA) were found at the level of 38.97% and 0.77% in the lipid from mantle. The most abundant fatty acid in squid mantle and tentacle was DHA followed by palmitic acid and eicosapentaenoic acid (EPA). Ozogul et al. (2008) reported similar results for *Loligo vulgaris.* Despite the fact that cephalopods contain very small amounts of fat, this organism is good sources of EPA and DHA content.

Table.3.3 Fatty acid composition of *Loligo duvauceli* (%)

Fatty acid	***L.duvauceli***
C 14:0 (Myristic acid)	2.36±0.12
C16:0 (Palmitic acid)	20.61±0.01
C18:0(Stearic acid)	6.14±0.11
C23:0(Tricosanoic acid)	1.1±0.03
∑SFA- Saturated Fatty Acid	**30.22**
C18:1(Oleic acid)	1.45±0.21
C20:1(Eicosenoic acid)	0.55±0.14
∑ MUFA-Monounsaturated Fatty acid	**2.02**
C20:4(Arachidonic acid) n-6	7.33±0.02
C20:5(Eicosapentanoicacid)n-3	13.86±0.12
C22:6(Decosahexanoic acid)n-3	46.43±0.14
∑PUFA-Polyunsaturated fatty acid	**67.62**
TOTAL	100

Values are expressed as mean±SD

3.4.4 Macro and micro minerals composition

The mineral content of *Loligo duvauceli* is presented in Table 3.4. Potassium 0.178±0.05 g/100g body weight was found as the major component of macro mineral in the edible portion of the sample, followed by Sodium and calcium. The most abundant micro-mineral in squid muscle wasmagnesium (158.019±0.23g/100g) followed by iron (3.341±0.06 g/100g) and zinc (1.036±0.05 g/100g). The result is similar with the finding of Thanonkaew et al. (2006). Lourenco et al. (2009) found that the main elements of common cephalopods were S, Cl, K, Na, P, Mg and Ca. Meanwhile, in juveniles and hatchlings of the cephalopod species showed a high content in Sulphur (Villanueva and Bustamante 2006). Cephalopods are carnivorous and active predators. In addition to that minerals absorbed from sea water by osmotic uptake through the gills and the body surface as the cephalopods live in hypo-osmotic environment. Minerals also absorbed by digestive gland as they swallow massive quantities of sea water during and after feeding (Wells and Wells, 1989). Minerals are essential for the maintenance of normal metabolic and physiological functions of cephalopods. (Lall,2002; Villanueva and Bustamante, 2006). Level of cadmium, lead and mercury were not detected in the study. Cadmium, lead and mercury were among element that could be harmful for organism (Nurjanah et al., 1999). Cephalopods are considered to be a vector for the transfer of cadmium to top marine predators (Bustamante et al., 1998; 2002). The digestive gland exhibited the highest cadmium accumulation compared with others organ, with food is likely the primary pathway bioaccumulation (Raimundo and Vale, 2008; Bustamante et al., 2002).No flesh sample showed cadmium and total mercury concentrations exceeding the peak permitted values of 2 mg/kg wet weight and 0.5 mg/kg wet weight respectively.

Table.3.4. Macro and micro minerals profile (wet basis) (g/100g) of *Loligo duvauceli*

Macro Mineral (g/100g)	***Loligo duvauceli***
Na	0.106±0.13
K	0.178±0.05
Ca	0.012±0.003
Micro Minerals (mg/100g)	***Loligo duvauceli***
Fe	3.41±0.06
Zn	1.36±0.05
Mg	158.19±0.23
Cu	0.16±0.06

Values are expressed as mean ±SD

3.5 Conclusion

Squid (*Loligo duvauceli*) is a nutritious aquatic commodity rich in protein with essential aminoacids and low in fat content as well as high in PUFA and essential minerals.Squid in iced condition may cause leaching of essential components mainly proteins responsible for the good organoleptic and nutritional characteristics. The extent of leaching affects the overall quality of the squid. Hence it is recommended to avoid direct contact with ice when transported from landing centers to domestic market or to processing plant.

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Chapter

4

BIOGENIC AMINES FORMATION IN WHOLE AND GUTTED SQUID (*Loligo duvauceli*) AND ITS RELATION TO SPOILAGE CHARACTERISTICS DURING STORAGE

Contents

4.1 Introduction

Like fish species, squid is also a highly perishable item and must be preserved immediately after harvest. The main factors responsible for the spoilage at ambient temperature are autolysis and bacterial spoilage. The rate and extent of the autolysis is considerably higher than bacterial spoilage and it plays a vital role in flavor development. Generally the rate of spoilage depends up on the temperature at which the squid is stored. By reducing temperature to about 0°C, both autolysis and microbial spoilage are reduced.

Squids, like most fish, are stored in ice during distribution in domestic market. As a result, the decline in the freshness occurs in iced condition with melting of water from the ice. There are few scientific studies providing information on quality changes of different species of squid after catch and during storage(Paarup, et al., 2002; Lapa-Guimaraes, Silva., 2002; Byun, et

al., 2000; Ohashi., 1991; Langille & Gill., 1984). Chemical indices for freshness evaluation of fish, crustaceans and mollusks are based on changes of non-protein nitrogen (NPN) components during the storage, such as volatile basic nitrogen (VBN) and trimethylamine (TMA). TMA and TVBN acceptability limits have been proposed for some squid species (Ke, et al., 1984). Other studies (Civera G, 1999; Paarup et al., 2002; Romo, 1996) have shown that acceptable limits are dependent on the species and storage conditions. In addition to the VBN analysis, the determinations of free tryptophan (Romo et al., 1996, Lapa-Guimaraes, et al., 2005; Romo, et al., 1996) urea (Romo, et al., 1996; Otsuka, 1992) hypoxanthine (Hx), Ammonia and Agmatine (Paarup, et al., 2002) have been evaluated as freshness indices in squid species. The change in the external appearance of cephalopods, specially related to a decrease in the skin reddish brown colour and shine, has been used for primary quality evaluation. Intensification and spreading of the pink colour of the skin with time, rather than the decline in skin colour is reported during spoilage of squid. (Guimaraes, et al., 2002; Lakshmanan, et al., 1993; Ke., 1984.)

Biogenic amines are important quality indices used in marine fishes. Biogenic amines (BAs) are non-volatile organic compounds, are formed in fishes by microbial decarboxylation of amino acids. The most important BAs are histamine, tyramine, tryptamine, putrescine, agmatine and cadaverine, are formed from free amino acids histidine, tyrosine, tryptophane, ornithine, arginine and lysine, respectively. Spermidine and spermine arise from putrescine (Zarei, et al., 2011). The biogenic amine content of fish depends on species, free amino acid content, and gut contents at death and also with the season. Biogenic amine (BA) production (histamine, putrescine, tyramine, and cadaverine) reported to correlate well with microbial load in squid (Prester,

2010). Histamine, one of the biogenic amines, has been known as the contributory toxin of scombroid fish poisoning (Onal, 2007), and histamine formation was reported to not be related to the activity of endogenous enzymes in fish, but due to histidine content in the fish muscle. Amines such as cadaverine and putrescine are also important in fish and fish products, since they have been shown to potentiate the toxicity of histamine (Shalaby, 1996). Biogenic amines are produced at very low levels in fresh fish and their formation is related to bacterial spoilage (Ozogul&Ozogul, 2006). Spermine and spermidine are usually the major amines present in fresh muscle at concentration of less than 10 mg/kg flesh, but depending on the fish species, the free amino acids present in the tissue and the conditions of exposure to spoilage bacteria (Onal, 2007). Only a limited number of studies have enclosed sensory, chemical and microbiological changes in squids during ice storage (Paulo Vaz-Pires, 2008, Paarup, 2002).The study elaborating the biogenic amine formed in whole and gutted squid during ice storage and to elucidate the role of evisceration in squid quality and also tries to correlate the biogenic amine formed with chemical, microbiological and sensory changes during storage.

4.2 Review of Literature

Freshness determines the quality of fish as food, both for domestic as well as industry. Biochemical, chemical, and sensory changes affect fish quality during handling and storage (Barret and others, 1965; Gill and others, 1987; Ehira and Uchiyama, 1986; Ke, et al., 1984 and Ke, et al., 1991). Different methods are universally applied to estimate freshness and quality of different fish species but a few studies have reported on the sensory, chemical and microbiological changes in chill-stored cephalopods (Ke, et al., 1984; Langille&Gill, 1984; Licciardello, et al., 1985; Yamanaka, et al., 1987;

Ohashi,etal., 1991; Byun, et al., 2000; Paarup, et al., 2002; Vaz-Pires&Barbosa, 2004; Lapa Guimaraes, et al., 2005). Further, indicators of decomposition in cephalopods are not well established as for fish. (Hurtado et al., 2001; Vaz-Pires & Barbosa, 2004; Barbosa & Vaz-Pires, 2004; Boumpalos&Lougovois, 2005; Ozyurt et al., 2006; Vaz-Pires&Seixas, 2006; Vladimiros, et al., 2007). Few studies have been directed at several species of squid on selected sensory attributes, mainly skin colour, which is a delicate quality parameter due the presence of chromatophores that easily break when exposed to direct contact with ice, leading to discoloration. (Ohmori, et al., 1975; Learson&Ampola, 1977; Botta, et al., 1979; Ke, et al.,1979; Lapa-Guimaraes, et al., 2002).

Once caught, cephalopods undergo very rapid protein degradation due to endogenous and bacterial enzymes. Such high proteolytic activity produces an increase in levels of muscle-derived nitrogen, hence favoring proliferation of degenerative flora and rapid decomposition (Hurtado, et al., 1998; Hurtado, et al., 1999). Parrup (2002) has investigated the sensory, chemical and bacteriological changes during ice storage of squid, *Todaropsis eblanae*. Combined sensory and biochemical studies have been conducted by Ke, et al., (1984, 1991), who elaborated a useful grading procedure for fresh *Illex illecebrosus* based on its content of total volatile bases, trimethylamine and free fatty acids, and correlated with organoleptic qualities. Yamanaka et al., (1987) related the production of biogenic amines with sensory qualities in *Todarodes pacificus*.The chemical, physical and the bacteriological parameters of the industrial samples of Loligo species and cuttle fish were studied by Lekshmanan, et al., (1993). Changes in several components and properties were examined to find appropriate indicators of freshness of common squid during storage at 0°C, 5°C and 10°C (Eijiohashi, 1991). Joseph et al (1977)

studied the quality of squid tube stored in ice. Mathew, et al., (1999) has investigated the distribution on non-protein nitrogenous extractives in the muscles of 41 species of marine fish of India including squid *Loligo duvauceli*.

The effect of storage conditions on sensory parameters, psychrophilic count, colour parameters of squid (*Loligo plei*) stored either in contact ice or in non-contact ice were studied by Lapa (2002). Yamanaka (1987) reported that agmatine appeared to be most useful as a potential index for freshness of common squid. Agmatine was detected in small amounts even in the fresh muscle and the concentration increased with storage time. Sagedhal, et al., (1998) has investigated the postmortem changes in Adenosine Tri phosphate (ATP) and related compounds in the mantle of squid *Illusargentines.* Changes in sensory attributes of raw and cooked squids were determined and compared with changes in *enterobacteriaceae* count for samples held in air at ambient temperature. Servet A tayeter (2011) studied the chemical composition of European squid (*Loligo vulgaris*) mantles and tentacles and the lipid oxidation during frozen storage at three different temperatures (-20º, -40º and -80 °C) were investigated. Civera (1999) has studied the chemical and microbial characteristics of cephalopods.The factors involved in the evaluation of sea squid found in Pakistan waters as a good source of protein was studied by Begum, et al., (1994).

The bacteriology of loligo species was studied by Joseph et al., (1997) and the presence of bacteria before and after the treatment with ascorbic acid on *Loligo duvaucelii* studied by Selvaraj (1991). Various handling and processing methods for Atlantic short finned squid (*Illex illecebrosus*) were studied by Ke, et al., (1991) including the effect of contact icing and non contact icing on the quality of squid. Yamasaki, et al., (1993) and Nishimura and Shinano, (1991) have studied the effect of trimethylamine oxide on squid

product with respect to the microflora, chemical properties and the growth of inoculated *Staphylococcus aureus*. Baldrati (1990) studied the handling, marketing and processing of cephalopods in Italy and the importance of cephalopods (cuttle fish, squid and octopus) in Italial seafood market. Longer storage of squid tubes and cuttlefish fillet in ice resulted in a noticeable decrease in NPN value (Joseph and Perigreen, 1988). The recently developed Quality Index Method (QIM) tables were used for sensory analysis of whole cuttlefish and shortfin squid.

Due to low volatility and lack of chromophors, biogenic amines are mostly analyzed by liquid chromatography (LC) with pre and post column derivatization and UV–visible or fluorescence detection (Onal, 2007). The drawbacks to these methods are time consuming, sample pretreatment that requires fluorescence derivatization, and liquid phase extraction. In this study, a simple, highly sensitive and direct analytical method with relatively easy sample preparation withoutderivatization is used involving Liquid chromatography (LC) coupled to mass spectrometry (MS).

4.3 Materials and Methods

4.3.1Sample collection and preparation

Freshly landed squid (*Loligo duvauceli*) were procured from a commercial fish landing centre, Kochi, Kerala. The average length and weight of the squid were 45-55cm and 145-160g respectively. Squids were immediately iced and transported to the laboratories. Time span from collection to receive at the laboratory varied from 30 to 45 minutes. Immediately after receiving they were washed in chilled potable water to remove excess ink, slime and any other extraneous material. The squid sample segregated in to two lots. One lot was eviscerated manually by squeezing out

the viscera, head and washing the mantle with chilled potable water while the second lot was retained as whole. Both the lots were iced (flake ice) in ratio of 1:3 in an insulated box and were placed in a thermo-statically controlled chill room (0 ± 2^0C) until unacceptable. Every second day of storage period, the boxes were drained, and additional fresh ice was added with alternating layers of squid and ice.The day the squid were captured was designated as 0^{th} day for the purpose of this study. Nine specimens were sampled every 3 day for the sensory, biochemical and microbiological analyses.

4.3.2 Sensory analysis

On each sampling day, whole and gutted squid were collected from the original lot and used for descriptive sensory evaluation by 8 trained panelists according to Quality Index scheme for shortfin squid (Vaz-Pires&Seixas, 2006). The main objective is to obtain a linear correlation between the sensory quality of the species expressed as the sum of demerit points and the time of storage in ice, predicting the freshness of a given fishery product (Hyldig& Green-Petersen, 2004; Hyldig& Nielsen, 1997, 2004; Larsen, et al., 1992). The Quality Index Method (QIM) is based on objective evaluation of the key sensory attributes of each fish species using a demerit points scoring system (Costell, 2002). Samples cannot be rejected based on a single parameter and minor differences in results of each one is not strong enough to influence overall QIM score (Luten & Martinsdottir, 1997). In the present study the sensory characteristics of squid are based on 4 quality attributes and 8 parameters (Appendix 1). The parameters were scored from 0 to 1, 0 to 2 or 0 to 3, depending on the different characteristics. The sum of the total scores for 16 demerit points, included 2 points for the skin appearance, 3 for the odour, 1 for the mucus, 2 for the flesh texture, 4 for the eye appearance and shape, and

4 regarded the mouth odour and appearance. Each panelist analyzed the squid samples individually and recorded his/her score for each parameter.

4.3.3 Chemical analyses

4.3.3.1 Biogenic amines (BA)

Biogenic amine contents of the squid muscle were measured according to Paleologos et al., (2004) method. Squid samples for Biogenic amine (BA) estimation were prepared by homogenizing 10 g minced squid with 25 mL of a 5% trichloroacetic-acid (TCA) solution in a homogenizer (Pro SC250) for 2 minute and filtering through a Whatman No. 1 filter paper. After centrifugation (Centrifuge- Bio fugeStratos, Germany) at 8000 rpm for 10 min at 4 ^{0}C, the supernatant was collected. The residue was extracted again with an equal volume of 5% TCA. Both supernatants were combined, and the final volume was adjusted to 50 mL with 5% TCA. The extracts filtered through 0.45 µm Millipore syringe filter and were stored at -20^{0}C until quantification with LC-MS/MS(Park, Kim, & Kim, 2010).

Chromatographic separations were performed by using an API 4000 Q-Trap^{TM} LC-MS/MS coupled with Waters Aquity UPLC system in Multiple Reaction Monitoring (MRM) using analyst 1.5.2 software. After dilution with methanol (ratio 1:10) the TCA extracted samples were directly injected (10µl) and analyzed without any further sample preparation. The chromatographic separation of seven BA (histamine, putrescine, cadaverine, tyramine, spermine, spermidine and agmatine) was achieved using Aquity BEH C18 1.7µm 2.1x50mm column and a gradient elution mounted at 55^{0}C using eluent A (Acetonitril with 0.005% Trifluoroacetic acid (TFA) and B (water with 0.005% TFA) with a flow-rate of 0.3ml/min. Chromatogram recordings and all calculations were performed on a Grid 325 SC computer (Grid, Greenwich,

Calif., U.S.A.). Quantification was done using a factor correction, calculated using histamine, putrescine, and cadaverine, agmatine, spermine, spermidine and tyramine at 1 mg/ml as standard.

4.3.3.2 Total volatile base Nitrogen (TVB-N)

The whole squid were washed, de-mantled, gutted and skin removed. The squid muscle was minced, using a common kitchen homogenizer, and maintained in ice until analyses during the same working day. 10g of minced sample was weighed and homogenized with 10% Trichloroacetic acid (TCA). The extraction was repeated 2-3 times and made up to the volume of supernatant to 50ml.

The Total volatile base nitrogen (TVB-N) was measured according to the Conway micro-diffusion method (Conway 1962). Total volatile base nitrogen (TVB-N) was estimated by pipette out 1ml of N/50 H_2SO_4 in to the inner chamber of the Conway diffusion apparatus. Pipette out 1ml of extract and 1ml of saturated potassium carbonate solution into outer chamber. The cover glass was slide in to position so that the entire unit is covered fully and ensured thorough mixing by rotating the unit. The entire unit was kept at room temperature for overnight. Excess acid in the inner chamber is titrated against 0.01N NaOH using Tachiro's indicator. A blank was also run simultaneously with 1ml of TCA instead of muscle extract. TVB-N was expressed as mg% of N/g sample.

$$\text{TVBN as mg\%} = \frac{0.28 \times (\text{blank} - \text{sample value}) \times 50 \times 100}{\text{weight of sample}}$$

4.3.3.3 Trimethylamine Nitrogen (TMA-N)

The procedure for the determination of TMA-N was the same as that of TVBN except that in the outer chamber along with 1ml of TCA extract, 1ml of

formaldehyde was added (Shewan 1971), and excess acid was titrated as previously described.The result was expressed as mg% of N/g sample

$$\text{TMA as mg\%} = \frac{0.28 \times (\text{blank} - \text{sample value}) \times 50 \times 100}{\text{weight of sample}}$$

4.3.3.4 pH

The pH analysis is done in squid muscle homogenized with 10 volumes of deionized water (w/v) by the method of Goulas and Kontominas (2005) usingpH meter (HI 2221 Calibration check pH / ORP Meter Hanna Instruments)

4.3.3.5 Nucleotide analysis

The concentration of nucleotide-degradation compounds (ATP, ADP, AMP, IMP, inosine, and hypoxanthine) in the squid muscle extract is determined using high performance liquid chromatography (HPLC) according to the method described by Ryder (1985). Sample extracts were prepared by blending 5g of minced squid with 25 mL 0.6 M perchloric acid at 0 °C for a minute. The homogenate was filtered through 0.45 μm Millipore syringe filter, and the filtrate immediately neutralized to pH 6.5 to 7.0 with 1M KOH. The neutralized extracts were filtered to remove insoluble potassium perchlorate and frozen in vials at -20 °C for subsequent analysis. The HPLC system equipped with a model L-7400 UV-Vis absorbance detector was used. A reverse-phase Lichrospher^{TM} C-18 stainless-steel column (25x cm 4 mm I.D.) having particle size of 10μm was used for separation. The mobile phase was 0.1M phosphate buffer pH 7.0 (0.04 M potassium dihydrogen phosphate and 0.06 M dipotassium hydrogen phosphate) at a flow rate of 1 ml/min. The eluent was monitored at 254 nm. Nucleotide standards mixture containing adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate(ADP), adenosine

5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino), and hypoxanthine (Hx) in 0.0276 μM 20 μl was used to calibrate the system.The % K value, calculated as the ratio of the sum of hypoxanthine and inosine to the total amount of ATP related compounds, was used as an index of fish freshness (Saito and others, 1959).

4.3.4 Bacteriology

The total mesophilic counts and psychrophilic count in squid muscle were determined according to the conventional aerobic plate count (APC) method (USFDA, BAM). Squid muscle was cut using sterile knife and forceps, pooled together and mixed by cutting in to very small pieces. Sample were transferred aseptically to a stomacher bag (Seward Stomacher circulator bag, Model No. 400, England) and homogenized with 225 ml sterile phosphate buffer for 30s at 230 rpm using a stomacher blender (Seward Stomacher 400CircularLondon, UK). Further tenfold serial dilution was prepared with sterile phosphate buffer. 0.5 ml of appropriate dilution was spread on the pre-set sterile plates for psychotropic counts and pour 44-46 ml melted plate count agar to sterile plate containing 0.2ml dilution for total plate count. The inoculated plates were incubated at 37 ^{0}C for 48 h and at refrigerated conditions for 7 days for total mesophilic counts and total psychotropic counts, respectively. After incubation microbiological data transformed into logarithms of the number of colony forming units (cfu g^{-1}).

4.3.5 Statistical analysis

The mean values and standard deviations of the experimental data from triplicates were calculated by Microsoft Office Excel 2003. Statistical analysis was performed in IBM SPSS Statistics version 20 and data reported as mean± standard deviation multivariate ANOVA at 5% level of significance was

performed to compare the treatment means. Turkey's multiple comparison test was used for post-hoc analysis.

4.4 Result and discussion

4.4.1Sensory analysis

In the present study the sensory characteristics of squid are based on the quality index scheme developed for raw squid consisted of 4 quality attributes and 8 parameters (Appendix 1). During the storage period, squid showed gradual and consistent changes for all the parameters of sensory evaluation, reaching a total score of 16 demerit points (figure 4-1). Rejection based on acceptability of external sensorial attributes and unpleasant odours of squid, occurred on10 days of storage in the case of whole squid and 14 days in the case of gutted squid.This compares well with the findings of Paulo Vaz-Pires (2008). Who demonstrated limited life for mollusks typically 9 days in the case of squid and 10 days for cuttle fish, after catch at low storage temperature of 2.5^0C.All parameters considered showed a clear variation within the first 8 days in ice. It was noted that the mantle of squid without deskinning exhibited the higher increases in mantle colour compared with the deskinned counterpart. Skin containing a number of chromatophores was most likely a major source of red or pink pigments, which were able to stain the mantle, especially as the storage time increased Lapa-Guimaraes, et al. (2002). Thanonkaew, et al. (2006) reported that the formation of yellow pigment in squid muscle could be due to non-enzymatic browning reaction occurring between aldehydic lipid oxidation products and the amines on phospholipids head groups. Eyes showed very rapid changes in the initial periods; while some othercharacteristics varied clearly towards the end of storage, e.g. mouth region odour, mucus and flesh texture. Cornea and pupil changes can be

irregular on both eyes of the same squid, probably due to physical damages caused, among other possible reasons, by contact with ice. All the parameters were considered to be useful to distinguish the freshness of squid. Slightly unpleasant odours started to be perceived around storage 4 to 6 day, but became unacceptable at day 10 in the case of whole squid, but the cleaned squid become unacceptable at the day of 14. This is mainly due to the colour of the skin which becomes pink and intense ammoniacal odour and flappy texture were the main parameters to define rejection. The developed scheme for squid with a maximum of 16 demerit points effectively assesses its freshness, which is correlated with ice storage time.

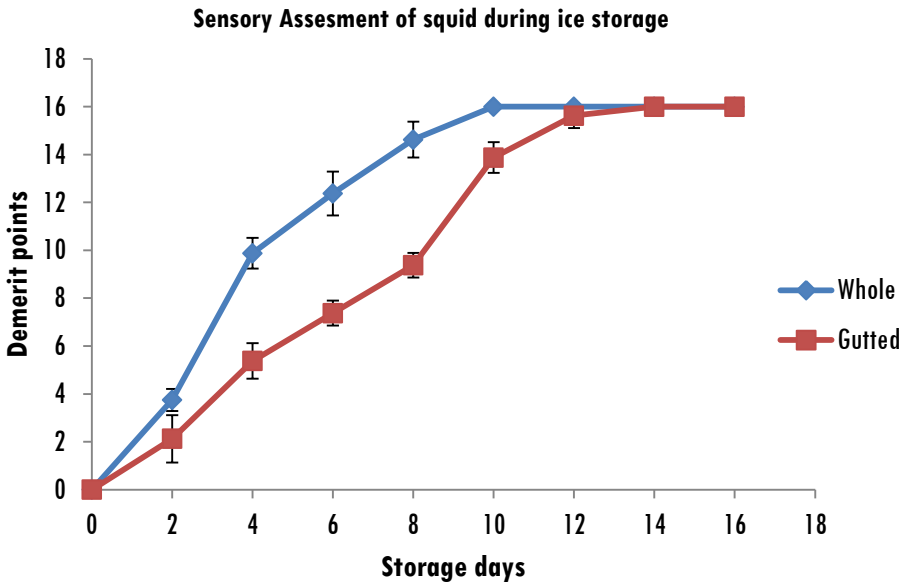
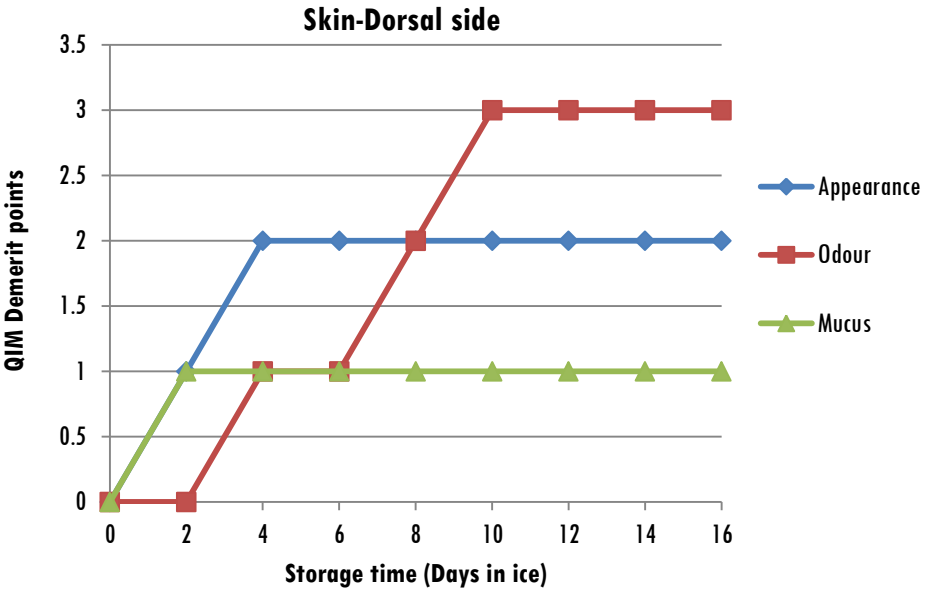
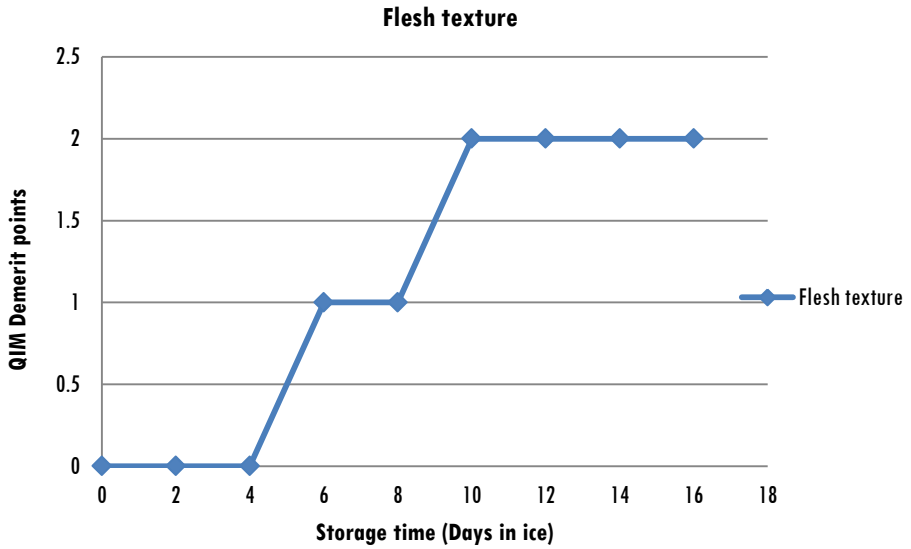


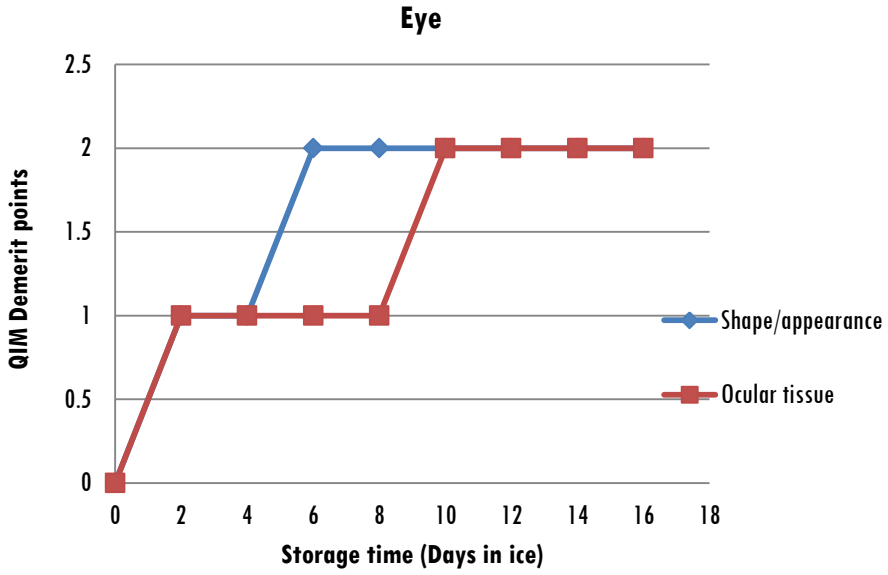
Fig.4.1 Changes in sensory demerit points of whole and gutted squid during ice storage. Mean values of triplicate samples; SDs are denoted as bars


Skin-Dorsal side
QIM Demerit points
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
Storage time (Days in ice)
Appearance
Odour
Mucus

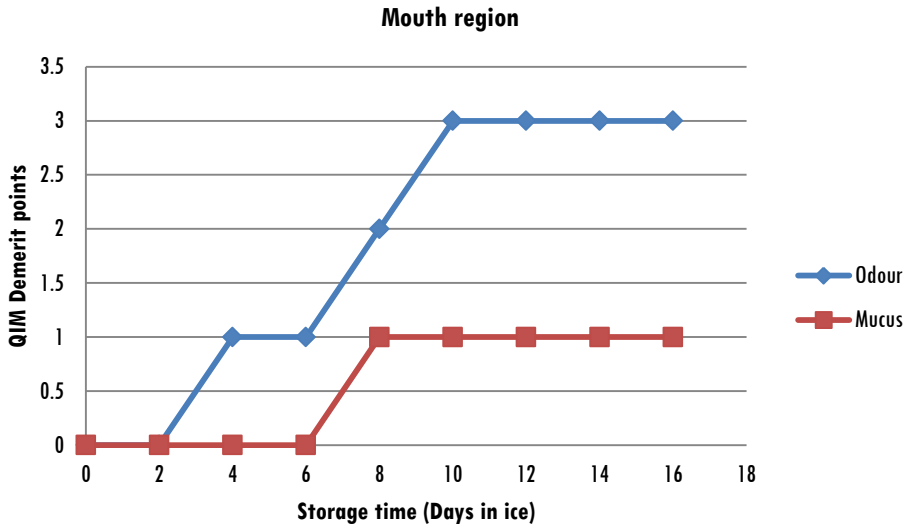
4.2(a)


Flesh texture
QIM Demerit points
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
Storage time (Days in ice)
Flesh texture

4.2 (b)

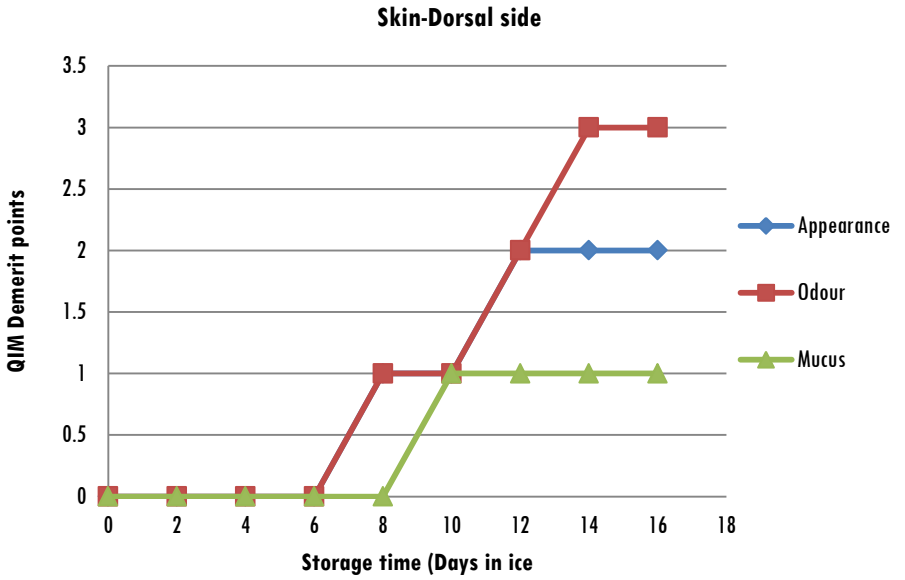


4.2 (c)

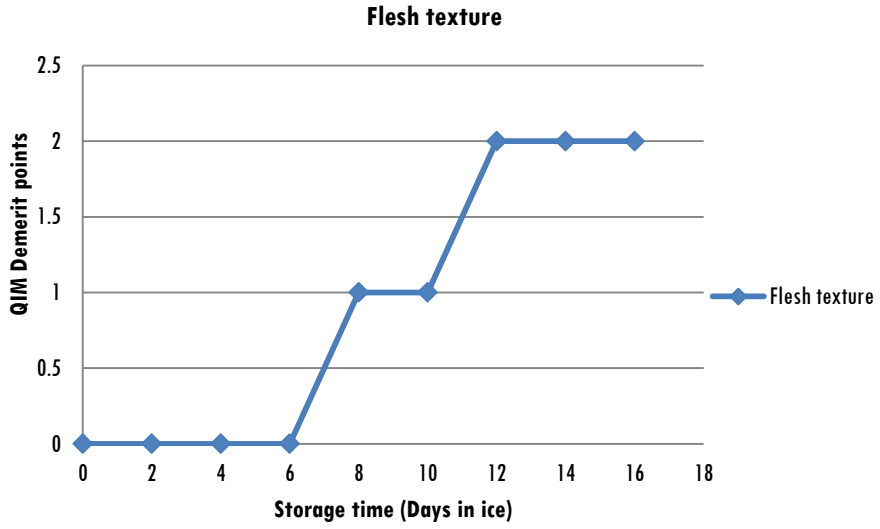


4.2 (d)

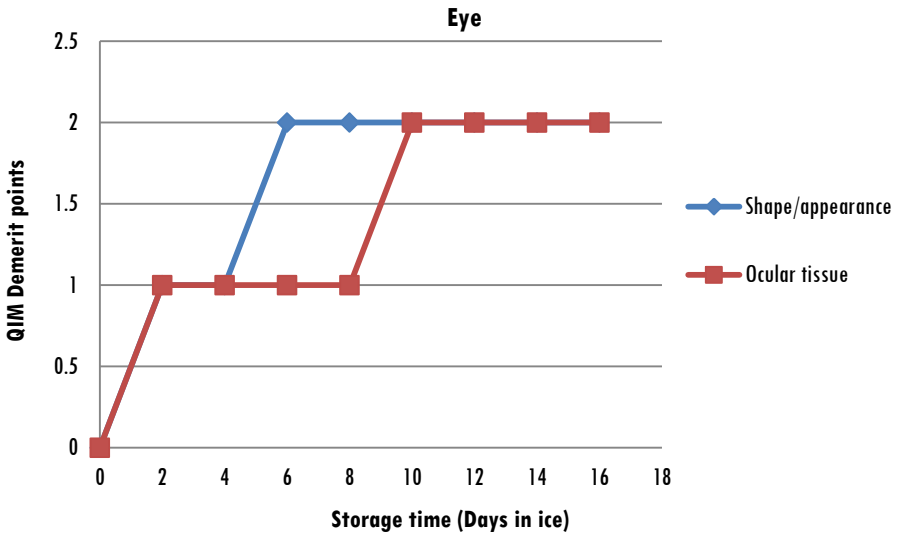
Fig.4.2. The mean demerit scores given on different storage days for each quality attribute of whole squid: (A) Skin; (B) Flesh texture; (C) Eye; (D) Mouth region.


Skin-Dorsal side
QIM Demerit points
3.5
3
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
Storage time (Days in ice
Appearance
Odour
Mucus

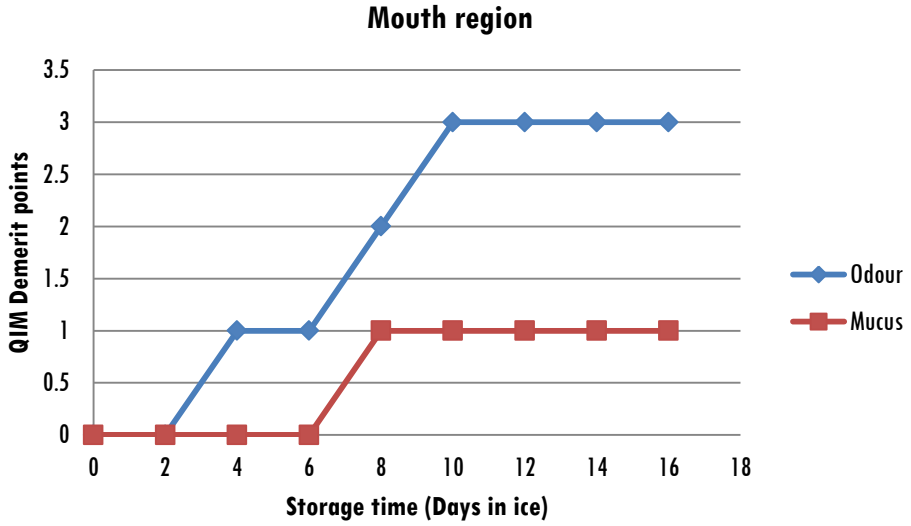
4.3 (a)


Flesh texture
QIM Demerit points
2.5
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
Storage time (Days in ice)
Flesh texture

4.3 (b)



4.3 (c)



4.3 (d)

Fig. 4.3. The mean demerit scores given on different storage days for each quality attribute of gutted squid: (A) Skin; (B) Flesh texture; (C) Eye; (D) Mouth region

4.4.2 Chemical Analysis

4.4.2.1 Biogenic amine (BA)

Table 4-1 show the production of biogenic amines in whole and cleaned squid muscles stored in ice. Seven biogenic amines were studied namely, putrescine (PUT), cadaverine (CAD), spermidine (SPE), spermine (SPER), histamine (HIS), tyramine (TYR) and agmatine (AGM) during the ice storage in both conditions.The analysis of variance of biogenic amines especially PUT, CAD, HIS, AGM, and TYRshowed significant difference between treatments ($p < 0.05$) and storage days ($p < 0.05$) and they increased with the storage period. Large changes in the contents of putrescine and cadaverine (Table 4-1) were observed throughout the storage period of both the squid samples. Putrescine, the amine responsible for putrid odour, was detected in small amounts at the stage of initial decomposition and increased rapidly at the stage of advanced decomposition. The concentration of putrescine in squid held in ice reached maximum of 210mg/kg and 120.8mg/kg respectively for whole and cleaned squid muscles on 16th day of storage period. The putrescine content of whole squid samples in ice was significantly ($p < 0.05$) higher compared to cleaned samples. This could be attributed to the effect of micro organisms on the skin and the gut in whole squid which is responsible for responsible for the formation of biogenic amines.

Cadaverine increased sharply as freshness decreases, and it could therefore be used to replace the traditional quality indices. Cadaverine levels reached the maximum level of 98.4 mg/kg for whole squid and 80.9 mg/kg in cleaned sample at the end of the storage (16 days) (Table 4-1). Significant differences were found ($p < 0.05$) in the levels of cadaverine among the two

treatments. Similar results were reported for other squid species *Loligo subulata* (Prester, et al., 2010), (Ozogul, 2008). Yamanaka, 1987 and Paarup, et al., 2002 suggest that rapid putrescine formation in decomposed squid (*Todarodes pacificus)* is related to early bacterial conversion of agmatine. Further putrescine showed no adverse health effects, high putrescine levels in decomposed squid could potentiate the toxic effects of histamine and tyramine (Lehane, 2000).

Among the biogenic amines, histamine is potentially hazardous and is understood to be the causative agent in scombroid poisoning (Arnold & Brown, 1978). In the present study, histamine was detected from 8^{th} day onwards in whole squid and 10^{th} day onwards in the cleaned sample. At the end of storage, whole squid samples presented a histamine level of 1.9 mg/kg and cleaned sample showed the level of 1.7mg/kg. However, the production of histamine is negligible compared to the scombroid fishes, because of the low level of amino acid histidine in squids (Yusuru, 1992). This is in confirmation with earlier report (Frank, et al., 1981), that fresh white fleshed fishes contain no or very little histamine. Ozogul et al., (2002) and Taylor, (1986) suggested that histamine could be a good quality index for scombroid fishes stored in ice. The European Community has set the maximum average histamine level for scombroid and scombroid-like fish to 100 mg kg^{-1} (EC 2005), whereas the US Food and Drug Administration has set this level to 50 mg kg^{-1} (FDA 1995). Currently there is no legal limit for histamine or other biogenic amines in any cephalopod species. The low level of histamine found in the study was consistent with some other studies on squid (Prester, et al., 2010;Yusuru,

1992) under different storage conditions. The low levels of histamine detected during the present experiment make intoxication problems in squid irrelevant.

No significant changes in the spermidine and spermine (Table 4-1) levels throughout the storage period in the two different conditions. The spermidine values were less than 7 mg/kg throughout the storage period in gutted samples, while in whole chilled samples the value slightly above percentage with increase in days of storage. Spermine showed high value in the range of 220 to 265mg/kg in both samples during storage. Paarup (2002) reported histamine and spermine never exceeded 2 and 5 mg 100 g^{-1}, in squid during ice storage. Ozogul et al. (2002a, 2002b) reported that ice storage inhibited the formation of these two amines in sardine and modified atmosphere packed in herring.

In both whole and gutted samples, the agmatine content was observed from 1st day onwards, which increased gradually during the storage.In cleaned squid the agmatine level increased up to 142 mg/kg (12th day) and decreased. In the case of whole increases was marginally higher and quickly 159 mg/kg in 8th day indicating the importance of cleaning of squids. The amino acid arginine is extremely abundant in free state in invertebrates (Ohashi, et al., 1991; Yamanaka, et al., 1987), in the form of arginine phosphate, a phosphate source for ATP synthesis (Contreras, 2002). Agmatine can be easily formed from arginine decarboxylation, which could be explaining the presence of agmatine in squid since the beginning of the storage. Agmatine produced could be, subsequently, converted in putrescine, spermidine and spermine, making any increase less noticeable PauloVaz-Pires et al. (2008). Yamanaka

(1987) reported agmatine appeared to be most useful as a potential index for freshness of common squid.

Tyramine is another important biogenic amine which has vasoactive and psychoactive properties along with its adverse reactions involving monoamine-oxidase inhibitor drugs. In the present study, tyramine appeared from fresh onwards and the production of tyramine concentration was low compared to the other biogenic amines. The concentration of 1.1 mg/kg was observed for both samples on the initial day, whereas a level of 2.2mg/kg and 2.8mg/kg were observed in cleaned and whole sample on 16th day, marginally higher for whole squid. Similar results were reported for carps (Krizek et al., 2004) and sardine (Ozogul, Polat, &Ozogul, 2004) stored under different conditions. Silla Santos (1996) reported that the tyramine content in decomposed squid exceeds the safety limit (100 mg kg-1) for human consumption. There was a significant correlation ($p < 0.05$) between the storage time and amine content (histamine, putrescine, tyramine, Agmatine and cadaverine) in squid samples.

Table: 4.1 Mean concentrations of biogenic amines (mg/Kg) in gutted and whole squid (*L.duvauceli*) during ice-storage

Changes in Biogenic amines (BA) in gutted sample (mg/Kg)							
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPERM**
0	ND	2.5±0.76a	ND	4.7±1.20a	1.1±0.30a	5.2±1.01cdef	265±2.01h
2	1.4±0.17ab	4±0.32a	ND	12.4±0.37b	1.2±0.12ab	5.6±1.11def	252±2.01f
4	5.2±1.04ab	6.5±1.20ab	ND	19.8±1.20b	1.3±0.03abc	3.4±1.12bcd	220±2a
6	11.4±1.22cd	11±1.05c	ND	24.3±1.20c	1.3±1.02abc	3.2±1.01ab	227±1.98bcd
8	24.4±0.51f	24.4±1.20d	ND	40±1.14e	1.4±0.03abcd	3.3±1.01ab	251±1.9f
10	35.8±1.50g	37.3±1.21f	1.3±0.14a	77.2±1.36g	1.7±0.01abcde	3.5±1.14ab	240±2.1de
12	102±1.53i	55.9±1.20h	1.3±0.20abc	141.6±1.20m	2.0±0.07cdef	2.5±1.01a	230±2.2b
14	115±1.51k	70.3±1.56j	1.6±0.03bcd	124.4±1.78h	2.1±0.03defg	3.9±0.59abc	245±1.08e
16	120.8±0.67m	80.9±1.29l	1.7±0.01cd	125.2±1.28i	2.3±0.03efg	3.2±0.59ab	244±2.31e
Changes in Biogenic amines (BA) in whole squid sample (mg/Kg)							
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPERM**
0	ND	2.5±0.76a	ND	4.7±1.20a	1.1±0.30a	5.2±1.01cdef	265±2.1h
2	3.6±0.26bc	2.7±0.33ab	ND	23.5±1.88b	1.2±0.02ab	5.1±0.11cde	257±2g
4	12.9±0.62d	11.9±1.27b	ND	65.2±1.36d	1.4±0.76ab	2.5±0.30cde	254±1.8i
6	21.2±1.34e	16.7±1.26c	ND	73.4±1.36f	1.3±0.24abcde	2.2±0.32cde	252±0.95f
8	37.6±1.32h	31.7±1.27e	1.1±0.07ab	159.8±0.31n	1.6±0.76bcdef	3.0±0.30def	243±2.7e
10	98.5±1.21j	48.4±1.87g	1.3±0.20abcd	135.7±2.70l	2.4±0.20efg	4.6±0.35cde	236±±2.5cd
12	163.3±1.34l	64.3±1.2i	1.6±0.14bcd	133.5±1.23l	2.2±0.04efg	6.8±0.24f	224±0.45a
14	195.6±1.28n	90.2±1.25k	1.7±0.20cd	129.8±1.36j	2.5±0.02fg	6.3±0.35ef	232±0.59bc
16	210.0±1.87o	98.4±1.27m	1.9±0.11d	129.5±0.82k	2.8±0.02g	6.7±0.02ef	241±0.23e

4.4.2.2 Total volatile base Nitrogen (TVB-N)

Total volatile basic amines (TVB) are one of the most widely used indicators of seafood quality. It is a general term which includes the trimethylamine (produced by spoilage bacteria), dimethylamine (produced by autolytic enzymes during frozen storage), ammonia (produced by the deamination of amino-acids and nucleotide catabolites) and other volatile basic nitrogenous compounds associated with seafood spoilage. Changes in the TVBN content in whole and gutted squid sample stored in ice storage are shown in Table 4-2.There was a significant difference in TVBN contents between treatments ($p < 0.05$) and days of storage ($p < 0.05$). Between treatments whole squid showed significantly higher vales than the cleaned one. TVBN contents of the squid mantle increased slowly in the initial days of storage after that a sharp increase were noticeable in the storage period after 6 days in whole and after 10 days in cleaned. The F value between methods (whole and cleaned) was 4841.05 and between days 18224.0.

Table 4-2 shows in the Initial day the TVBN content was 8.3 mg/100 g, which is increased to 32.7 mg/100 g in cleaned squid and 42mg/100 g in whole squid after 16 days of storage at low temperature. These results were similar to those found for other squid species (Lapa-Guimaraes et al., 2005; Ohashi et al., 1991; Vaz-Pires et al., 2008; Prafulla et al. 2000). Mohan et al (2004) reported that in Loligo without GMP, TVNexceeded 30 mg % on 4th day of storage while storage under GMP andwithout direct contact with ice, TVN remained within the limit even up to 8 days ofstorage. Tanikava et al., (1954) observed mustiness in raw squid, which indicated the spoilage when TVN reached 30mg/100g.TVBN has been considered as a useful spoilage indicator in different species of fish, having little use as a freshness index in cephalopods (Civera et al., 1999; Ohashi et al., 1991; Yamanaka et al.,

1987).It has also been reported that the rejection limit for TVB-N in fish varied with species and processing condition (Dalgaard, 2000).

4.4.2.3 Trimethylamine Nitrogen (TMA-N)

The changes in TMA content during ice storage by whole and gutted squid are given in Table 4-2 gives the ANOVA of TMA in both methods of ice storage. There was significant difference in TMA between treatments ($p < 0.05$) and between days $p < 0.05$). In the study the content of trimethylamine (TMA) was low(4.31mg/100g) in the initial days of storage in whole and gutted squid sample which increased exponentially from 2nd day onwards to16.43 mg/100 g and 22.7 mg/kg in the gutted and whole lot respectively at the end of the storage day. It was also noted that the content of TMA and TVBN in the whole squid samples was relatively higher as compared with the cleaned samples during the storage period, explaining the higher rate of bacterial and enzymatic decomposition in whole sample. These results were similar to those found for other species of squid (Jeyasekaran G. 2010; Paulo Vaz-Pires 2008; Lapa- Guimaraes et al., 2005) of storage. Ruiz-Capillas et al. (2002) reported low levels of TMA-N contents were observed in volador (*Illex coindetii*), pota (*Todaropsis eblanae*) and octopus (*Eledonecirrhosa*) in the initial days in ice. Paarup et al. (2002b) observed very high level of TMA-N content (above 40 mg/100 g) at the end of storage. Rate of increase in TMA-N in marine fish varied considerably from species to species (Huss 1988). The valuesexceeding 3 to l0mg of TMA per l00g of sample is the limit of acceptability in fatty fish (Woyewoda and Ke, 1980) and for fin fish species10-12mg/100g is reported to be the limit of acceptability. Mohan et al (2004) reported that in loligo processed without GMP, TMA crossed the limit of 10mg% and became unacceptable on the 3rd day of storage and in samples

processed under GMP on 6th day of storage and without direct contact on the 8th day of storage. In the present study TMA crossed the limit of acceptance on 10^{th} and 12^{th} day of storage for whole squid and gutted squid respectively.

TMA production in fish and shellfish occurs due to the action of bacterial enzymes on the trimethylamine oxide (TMA-O). A lower content of TMAO-N were found in common squids. However, a level of 224 mg of TMAO-N in fresh squids of the Loligo genera was reported by Contreras1994. Liston (1980) and Sikorski, et al., (1994) reported that the TMA production correlates well with the bacterial growth and TMA is the main compound responsible for the smell of spoiled fish. The fast increase of TMA and TVBN in second week of storage was certainly responsible for the unpleasant odours that started to be perceptible by the sensory assessors on day 8 and became unacceptable at day 10 the whole squid sample and at day 12 the gutted squid sample. This fact indicates that sensory rejection occurred when the bacterial effects on the cephalopods quality became more noticeable.

4.4.2.4 pH

The pH of muscle tissue of live squid is close to neutrality (Huss, 1995). During the later post-mortem changes, pH is more or less constant or slightly increased due to the formation of basic compounds (Huss, 1988). Even though the changes in pH are generally rather small, they have great technological importance. The post-mortem pH is the most significant factor influencing the texture of the meat and the degree of "gaping", means the rupture of the connective tissue (Huss, 1988). The reasons for this is that even minor changes in pH drastically affect the properties of the connective tissue and the net surface charge on the muscle proteins is reduced causing them to partially denature and loose some of their water holding capacity (Huss, 1995).

In the present study, the Table 4-2 shows postmortem muscle pH varied from 6.1 to 7.8 for whole squid and 6.1 to 7.6 for cleaned squid during the 16 days of storage period. There was no significant difference in pH in 1st and 2nd days of storage, which however in both conditions increased to gradually ($p < 0.05$) with time. The pH in the whole squid samples was relatively higher as compared with the cleaned samples during the storage period, explained by the increases in TVB, and TMA contents and other bacterial metabolites in the muscle (Howaida, 2010; Sikorski 1990). Similar results were reported for squid (*Loligo formosana*) (Rattana Sungsri-in, 2011), giant squid (*Dosidicus gigas*) (Marquiz-Rios, 2007) and squid (*Loligo duvaucelli*) (Jeyasekaran G. 2010. Paarup et al. (2002b) reported a pH range from 6.8 to 7.8 in squid mantle (*Todaropsis eblanae*) during storage at 4°C.Yamanaka et al. (1987) reported that the rapid increase in pH in common squid (T. pacificus) was noticed on storage at 15°C, compared with at 0°C and 3.5°C. Prafulla et al. (2000) observed that the pH of squid and cuttlefish muscle stored in ice did not vary significantly.

Table: 4.2 Changes in pH, TMA (mg/100 g) and TVBN (mg/100 g) in gutted and whole squid (*L.duvauceli*) during ice-storage

Gutted squid (*L.duvauceli*)				Whole squid (*L.duvauceli*)			
Days	**pH**	**TMA**	**TVBN**	**Days**	**pH**	**TMA**	**TVBN**
0	6.1±0.1^{a}	4.31±0.12^{a}	8.37±0.08^{a}	0	6.1±0.10^{a}	4.31±0.12^{a}	8.37±0.08^{a}
2	6.2±0.1^{ab}	5.21±0.49^{b}	9.17±0.09^{ab}	2	6.3±0.10^{abc}	5.33±0.10^{b}	10.20±0.08^{bc}
4	6.4±0.1^{bcd}	5.81±0.12^{c}	10.61±0.63^{c}	4	6.6±0.12^{de}	6.19±0.01^{d}	12.86±0.07^{d}
6	6.5±0.06^{cde}	6.28±0.08^{cd}	10.9±0.33^{d}	6	6.7±0.06^{ef}	7.12±0.15^{f}	13.46±0.10^{d}
8	6.7±0.10^{ef}	6.55±0.13^{e}	12.4±0.63^{e}	8	6.9±0.06^{f}	7.45±0.02^{g}	16.41±0.07^{g}
10	6.87±0.06^{f}	7.85±0.12^{h}	16.17±0.65^{f}	10	7.1±0.10^{g}	10.36±0.07^{i}	27.26±0.55^{i}
12	7.13±0.12^{g}	10.63±0.11^{i}	22.1±1.46^{h}	12	7.4±0.11^{h}	12.61±0.02^{j}	31.36±0.07^{k}
14	7.43±0.06^{h}	13.51±0.10^{k}	27.64±0.99^{j}	14	7.6±0.10^{hi}	16.21±0.01^{l}	36.41±0.07^{l}
16	7.63±0.01^{hi}	16.43±0.12^{l}	32.73±0.35^{k}	16	7.8±0.10^{i}	22.67±0.00^{m}	41.19±0.38^{m}

4.4.2.5 Nucleotide changes

The ratio between inosine and Hx to total nucleotide degradation provide a clear indication of spoilage in fish and shell fish. Postmortem degradation of ATP in fish muscle occurs due to endogenous-enzymes activity through the formation of ADP, AMP, IMP, INO, and Hx (Church 1998; Perez-Villareal and Pozo 1990; Ehira and Uchiyama, 1986). And there exists differences in the nucleotide-degradation patterns from species to species (Church 1998; Ohashi et al., 1991; Murata and Sakaguchi 1986; Ehira and Uchiyama 1986). In the present study the nucleotides degradation compounds formed during iced storage (Table 4-3) of squid followed a different pattern as compared with that from fish species. The adenosine 5'-triphosphate (ATP) content at 0^{th} day was 0.54µmol/g which completely depleted on 2^{nd} day in chilled condition in gutted squid and in the case of whole squid complete loss of ATP was detected on initial day itself. While at the same time Hx the predominant catabolite concentration increased from 273µmol/g on the initial day to 458µmol/g on the 4^{th} day and followed by a reduction pattern reaching 19µmol/g on day 16 in whole squid. Other lot also shows the same pattern except that the concentration of each nucleotide varied. Rapid ATP degradation in several squid species has been reported (Marquez-Ríos, 2007; Yoshioka et al., 2003; Shirai and others 1997; Yokoyama et al 1994). Marquez-Rios et al. (2007) reported that ATP was almost completely depleted at 24-h post catch from 6.54 to <1 µmol/g. At the same time, Hx was the predominant nucleotide with a concentration of 4µmol/g which reached 6.85µmol/g on day 16. Prafulla et al. (2000) also observed an increasing trend of hypoxanthine content in whole cuttlefish stored in ice. Further, ADP and

AMP concentrations at 0^{th} day were 25µmol/g and 58µmol/g respectively in gutted squid sample and 18µmol/g and 35µmol/grespectively in whole squid, while for the rest of the compounds (IMP & HXR) were below the detection limit on the initial day of ice storage (Figure 7 & 8), which agrees with the earlier report (Howgate, 2005). Both the nucleotide did not show any consistent trend throughout the storage period (Tables 4-4).

Unlike usual fish meat, K value in squid muscle changed very quickly, and did not show a consistent trend;and hence monitoring adenosine 5'-triphosphate (ATP) degradation and calculating the K value as freshness indexes for squid was little difficult. Different studies indicated that the ATP degradation proceeded with time and disappeared almost completely in approximately 24 h (Ohashiet al., (1991); Saito and others (1959); Yokoyama and others (1994); Shirai and others (1997); Yoshioka et al., (2003). Oacno-Higuera et al., (2006) had proposed AMP as an alternative freshness indexes for mollusk due to lack of linearity of the K value in contrast to fish. As shown in table, ATP degraded rapidly into Hx; however, different from our results, Yoshioka and others (2003) reported a predominant accumulation of AMP instead of Hx in *Todarodes pacificus* at 24h postmortem, indicating the possibility of using AMP as possible spoilage indicator.

Table: 4.3 Changes in ATP and its degradation products ingutted and whole squid (*L.duvauceli*) during ice-storage (n = 3)

Gutted squid (*L.duvauceli*) (µM/g)						
Days	**ATP**	**ADP**	**AMP**	**IMP**	**HX**	**HXR**
0	0.5±0.81	25.7±1.01	58.6±0.12	ND	273.6±1.21	ND
2	ND	19.9±1.0	28.0±1.02	ND	368.5±1.05	16.2±1.21
4	ND	13.9±0.23	18.7±0.21	0.8±0.01	431.9±0.84	19.1±1.14
6	ND	11.2±0.07	15.6±0.54	0.8±0.04	356.2±0.74	15.4±1.25
8	ND	9.3±0.05	9.6±0.80	0.9±0.06	245.0±0.32	2.1±0.07
10	ND	5.3±1.02	3.4±0.50	1.5±0.12	136.4±1.17	1.1±0.41
12	ND	4.7±0.78	3.2±0.14	2.5±0.04	108.8±0.09	0.8±0.14
14	ND	3.4±0.45	2.8±0.12	2.5±0.14	77.9±0.58	0.8±0.05
16	ND	1.7±0.15	0.8±0.14	2.9±0.07	52.4±0.50	0.5±0.06
Whole squid (*L.duvauceli*) (µM/g)						
Days	**ATP**	**ADP**	**AMP**	**IMP**	**HX**	**HXR**
0	0.5±0.81	25.7±1.01	58.6±0.12	ND	273.6±1.21	ND
2	ND	17.2±0.04	23.3±0.14	0.2±0.74	365.3±0.13	18.3±0.04
4	ND	14.3±0.05	16.3±0.14	0.4±0.56	458.2±0.015	21.5±0.074
6	ND	12.4±0.45	13.6±0.25	0.5±0.14	335.2±0.07	17.5±1.02
8	ND	8.3±0.78	5.4±0.23	0.7±0.78	154.2±0.05	1.5±1.51
10	ND	2.6±0.05	1.5±0.27	1.3±0.24	65.2±0.41	0.5±0.07
12	ND	0.9±0.41	1.3±0.05	2.9±0.63	32.1±0.06	0.2±0.06
14	ND	0.7±0.64	0.2±0.08	1.3±0.45	23.1±0.07	0.2±0.15
16	ND	0.4±0.12	0.2±0.47	1.1±0.06	19.7±0.78	0.1±0.06

4.4.3 Bacteriology

4.4.3.1 Total Plate Count (TPC)

The analysis of variance of total bacterial count (TPC) in logarithmic values showed significant difference between treatments ($p < 0.05$) and days of storage ($p < 0.05$), though no significant difference was noticed between 0^{th} to 2^{nd} day (Table 4-3). The whole squid in ice showed significantly higher bacterial growth than the gutted samples. In gutted samples there was a

reduction of TPC in the first two days and then the number gradually increased with storage days.

Whole Squids, analyzed in initial day after catch, showed a TPC value3.8±0.41 log cfu/g of sample. From 4^{th} to 16^{th} day, a steady increase in bacterial load was seen and reached to 5.6±0.06 log cfu/g at rejection10^{th} day). Initial reduction in TPC could be due to leaching and chilling effect of ice on bacteria. Similar trend was seen in the case of gutted sample also. In gutted samples the bacterial load was 5.5±0.04 log cfu/g at rejection (12^{th} day).These data confirm results of other studies in different species of squid. (Lapa-Guimaraes, 2005; Lapa-Guimaraes, 2002). TPC increased with duration of storage in ice time and decreased the quality of cephalopod (Civera, 2000). *Shewanella putrefaciens*, *Pseudoalteromonas* sp and *Pseudomonas sp* dominated in spoiled gutted squid (Paarup, 2002). Paarup, 2002 also reported that in squid loligo species, TPC analysis revealed an initial bacterial flora lower around $8.3x10^3$ and $3.6x10^5$cfu/cm^2 at rejection (15th day).

It is well known that the spoilage process in cephalopod is different from fish due, among many less clarified reasons, to thinner and fragile skin, nutritional composition more favorable to enzymatic degradation, shorter and less pronounced rigor mortis, and on set of autolytic degradation (Hurtado et al., 1999; Vaz-Pires & Seixas, 2006). The results obtained in this work suggest that these differences exist and are normally observed. Paarup et al. (2002b) reported that the aerobic plate count of squid mantles stored at 4°C increased from an initial level of 4 to 7 log cfu/g, when it was sensorially rejected. Pires and Barbosa (2004) found a bacterial load of 5–6 log cfu/cm^2 in octopus (*Octopus vulgaris*) stored in crushed ice at the point of rejection. Prafulla et al. (2000) observed that the total plate count did not reach 7 log cfu/g. The relatively low TPC (as measured in this work) at the point of rejection (10^7-

10^9cfu/g) was reported for many fish and fish products (Huss, et al 1997; Olafsdottir et al., 1997) which support the common idea that enzymatic action is more rapid and effective in cephalopods (Hurtado et al., 1999; Hurtado, Montero, &Borderıas, 2001; Lapa-Guimaraes et al., 2002; Ohashi et al., 1991).

Table: 4.4 Changes in Total plate count and psychrophilic count ingutted and whole squid (L.*duvauceli*) during ice-storage (n = 3)

Days	TPC log cfu/g	Psy cfu/g	Days	TPC log cfu/g	Psy cfu/g
Gutted squid (*L.duvauceli*)			**Whole squid (*L.duvauceli*)**		
0	3.8±0.06^{a}	2.6±0.07^{a}	0	3.8±0.41^{a}	2.6±0.11^{a}
2	3.7±0.56^{a}	2.5±0.12^{a}	2	3.8±0.07^{abc}	2.6±0.12^{a}
4	3.9±0.23^{ab}	3.3±0.14^{b}	4	4.5±0.06^{abc}	3.2±0.04^{b}
6	4.1±0.12^{abc}	3.6±0.11^{cd}	6	4.7±0.15^{abc}	3.4±0.07^{bc}
8	4.5±0.05^{abc}	3.9±0.41^{de}	8	4.9±0.04^{abc}	3.9±0.05^{e}
10	4.9±0.13^{abc}	4.7±0.06^{f}	10	5.6±0.06^{abc}	5.1±0.09^{g}
12	5.5±0.04^{abc}	5.2±0.08^{g}	12	5.9±0.34^{bc}	5.5±0.01^{h}
14	5.9±0.06^{bc}	5.9±0.08^{h}	14	6.3±0.21^{c}	6.1±0.7^{j}
16	6.2±0.91^{c}	6.0±0.04^{ij}	16	6.4±0.74^{c}	6.2±0.03^{j}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

4.4.3.2 Psychrophilic count (PC)

During ice storage, the continuous increase in psychrophilic count was observed in whole and gutted squid sample. The analysis of variance of psychrophilic count showed significant difference between treatments ($p < 0.05$) and days ($p < 0.05$). The whole squid in ice showed significantly higher growth rate than the gutted sampleTable 4-3. The initial psychrophilic count was 2.6±0.11 log cfu/g, which increased to 5.5±0.01 log cfu/g in whole squid and 5.2±0.08 log cfu/g in gutted squid after 16 day of ice storage. Rattana Sungsri-in et al. (2011) found that psychotropic counts in squids (*Loligo formosana*) increased from $2.9x10^2$ to $1.5x10^6$ cfu/g after 16 days of iced storage. Lapa-Guimaraes, et al., (2005) reported that psychrophilic count

increased from $1x10^4$cfu/g at the beginning of storage to 5 $x10^6$and $4x10^6$cfu/g in contact and non-contact iced condition, respectively, after 12 days of storage. Lapa-Guimaraes, et al., (2002) also observed a psychotropic bacterial count of 6 log cfu/g in squid (*Loligo plei*) after 16 days of storage in ice.The increases in psychrophilic count were in agreement with the increases in TVB, TMA and BA in *loligo duvauceli*, in their study.

4.5 Conclusion

In a developing tropical country like India, where distribution of frozen fish in domestic marketis not popular due to lack of cold chain facility, short term preservation of fish by icing has gained importance. Average consumers prefer fresh fish to iced/frozen fish even if both show the same degree of spoilage. Chemical and physical qualities play vital role in grooming the quality of a product. Since the spoilage of squid starts immediately after the death which largely dependent on the temperature conditions and gut contents at death;sooner the squid cooled the better will be the quality and shelf life. The results of the study indicated that the gutted squid (*Loligo duvauceli*) stored in ice had longer shelf life and better quality when compared to the whole squid.TMA and TVBN, the chemical indices traditionally used, showed an increasing trend during the days of storage in whole squid and slow increase in gutted squid, serving a little to estimate squid freshness andremaining shelf life. Biogenic amines especially PUT CAD, AGM, TYR and HIS, on thecontrary, could be useful freshness indices for *Loligo duvauceli*, because their contents progressively increased from the beginning of storage in both treatments. This study has provided new information on the spoilage markers of squid in different storage conditions like whole and gutted in ice storage. The results indicated that deterioration in the quality of the squid is highly depended on the condition of the storage and time. Whole and

gutted squid sample in ice storage, the level of histamine and tyramine did not exceed limits and has no adverse health effects. But there was a significant correlation between the treatment, storage time and amine content (histamine, putrescine, tyramine, Agmatine and cadaverine) in squid samples, indicating the possibility of using them as quality makers, spoilage makers in squid.

Based on the results of the various bacteriological, physical and chemical parameters it is concluded that when squid (*loligo duvauceli*) whole and gutted stored on ice keeps the characteristics suitable for human consumption up to 10 and 12 days respectively. Generally, biogenic amines production in squid can be controlled by strict use of hygiene practices in both raw material and manufacturing environments,which contribute to reduction/inhibition of spoilage microorganisms.

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Chapter

5

THE EFFECT OF STORAGE TEMPERATURE ON THE PRODUCTION OF BIOGENIC AMINES IN SQUID (*Loligo duvauceli*)

Contents

5.1 Introduction

Globally, there is an increasing demand for squid as food (Paarup, et al., 2002). Squid is a good source for a digestible animal protein, healthy polyunsaturated fatty acids, minerals and vitamins that support the biochemical processes in the human body (Lapa-Guimaraes, et al., 2005). However, it is high perishable compared to other sea-foods and spoilage begins faster in squid particularly in tropical countries due to environmental conditions (Vladimiros, et al., 2008). High ambient temperature supports squid spoiling within 6-12 hours after death leading to reduction in quality (Lakshmanan, et al., 1993). Because of this squid need to be utilized quicklyafter catch or subjected to processing like chilling, freezing, canning, smoking and drying to delay the spoilage and to extend the shelf life to be fit for human consumption (Ke, et al., 1991). This demands hygiene handling and

processing under controlled temperature, otherwise spoilage might increase very rapidly (Le Bihan, et al., 2006). A lot of studies have investigated the relation between storage temperature, time and changes in squid quality (Ohashi, et al., 1991; Lakshmanan, et al., 1993). The quality changes are strongly influenced by many factors, the most important one being temperature. Tropical climate in India, with an average temperature range between 25-40^{0}C is suitable for rapid spoilage of squid. In squid catching vessels and/or squid-processing facilities, squid often exposed to inappropriate hygienic procedures during handling on onboard fishing vessels or in production units which possibly lead to increase the loss in freshness of squid. Considering the importance of keeping squid quality high, this work was designed to study the effect of different storage temperatures, the temperature to which squid is exposed during different handling and processing stages (-18±2°C, 0±2°C, 4±2°C, and 30±2°C) on squid quality.

Besides, the seafood-borne disease is on increase world over, and there is a special requirement to reduce the food safety hazard in the product meant for human consumption (Ohashi et al., 1991). Histamine, one of the naturally formed biogenic amines, has been reported to be the cause of an outbreak of seafood poisoning in many countries (Onal, 2007; Shalaby, 1996). The formation of biogenic amines in aquatic products depends on many factors, such as the contents of free amino acids, contamination of certain bacteria, the presence of bacterial amine decarboxylases, and the storage conditions of the aquatic products. In fact, high levels of biogenic amine in aquatic products are related to inappropriate and inadequate preservation leading the formation of biogenic amine through the microbial decarboxylation of amino acids by various specific amine-forming enzymes (Onal, 2007). Therefore, the contents of biogenic amines in aquatic products may strongly be influenced by the

freshness of raw materials and the environmental storage conditions, especially storage temperature and time (Ozogul&Ozogul, 2006).

The main objective of the present study is to evaluate the production of major biogenic amines in relation to chemical and sensory changes in Indian squid (*Loligo duvauceli*) stored in different storage temperature and the duration of storage. The study also investigates the potential usefulness of biogenic amines as a freshness quality index.

5.2 Review of Literature

Many studies have been carried out to examine the effect of temperature and storage conditions on quality changes in squid particularly with reference to changes in TMA, TVBN, pH, BA especially agmatine. (Lapa-Guimaraes, et al., 2002; Lapa-Guimaraes, et al., 2005; Ohashi, et al., 1991; Yamanaka, et al., 1987). However, reports state that there are differences in the formation of amines in squid during ice storage and such differences are related to the type and level of microflora present in squid (Lopez-Sabater, et al., 1996). The increasein the formation of agmatine in squid was observed as a function of storage time at 10^0C (Ohashi, et al., 1991). There are reports indicating increases in free amino acids, especially arginine and ornithine, during storage and could be used as early freshness indicators (Ohashi, et al., 1991).

Biogenic amine production in different fish sample at different storage temperature showed 2-20 times higher levels of PUT, CAD, SPD and HIM in herring and mackerel stored at 10^0C than those of samples stored at 20^0C for the same storage period (Klausen& Lund, 1986).It was reported that the level of HIM in Indian mackerel (*Rastrellinger kanagurta*) stored in ice (0-5^0C) for 16 days was 8.3 mg kg-1, while the level increased to 962 mg kg^{-1}on storage at

27^0C for 20 h Chong, et al., (2014). It has been proved that the formation of BA in aquatic products may be strongly influenced by the storage temperature and the mesophilic bacteria and the psychrophilic bacteria in the fish (Moini, et al., 2012). Santos, et al., (1986) reported that temperature did not significantly influence tyramine formation in anchovies though refrigeration temperatures delayed the production of amine. Diaz, et al., (1992) found that histamine and tyramine concentrations increased with the time and storage temperature of chihua cheese. Putrescine biosynthesis by *Enterobacter cloacae* was detected at 20 ° C after 24 h of incubation but not at 10°C, and *Klesbsiella pneumoniae* showed less extensive cadaverine production at 10°C compared to 20° C. Further it is reported that histamine production slowed at 10° C and nearly terminated at 5° C. This is attributed to the slow growth of histamine producing bacteria at low temperatures. No histamine was formed by *Pseudomonas morganii, Pseudomonas vulgaris, or Hafitia* strains after one month of incubation at 1° C (Halasz et al., 1994). Similarly, Klausen and Lund, (1986) reported that amine contents were temperature dependent and were two to twenty times higher at 10° C compared to that at 2° C in both mackerel and herring. Most of the studies demonstrated that there is direct relationship between biogenic amine formation and time and temperature of storage and temperature abuse in highly perishable food items is the main cause of biogenic amine production and the food safety concern.

5.3 Materials and Methods

5.3.1 Sample preparation

Freshly caught squid (*Loligo duvauceli*) were obtained from Thoppumpady Landing Center, Cochin, India. Samples were iced immediately and transported to the CIFT laboratory within 30 minutes. Samples were

divided into 4 groups, iced and stored at different storage temperatures-18±2°C, 0±2°C, 4±2°C, and 30±2°C. After the initial analysis at zero time samples stored at different conditions were analyzed at different intervals. Samples were monitored periodically during storage conditions. Samples stored at 30±2°C were taken for analyses every 6-hour, samples stored at 0±2°C, and 4±2°C was analyzed onalternate days, at -18±2°Cstored samples were analyzed at 2 months intervals. The analytical methods for biogenic amines (PUT, CAD, HIS, AGM, TYR, SPER, SPERMD), chemical evaluation (TMA, TVBN, pH) and microbiological evaluation are given chapter 3, section 3.3.1

5.3.2 Statistical analysis

The mean values and standard deviations of the experimental data from triplicates were calculated by Microsoft Office Excel 2003. Statistical analysis was performed in IBM SPSS Statistics version 20 and data reported as mean± standard deviation. Multivariate ANOVA at 5% level of significance was performed to compare the treatment means. Turkey's multiple comparison tests was used for post-hoc analysis.

5.4 Results and Discussion

5.4.1 Sensory analysis

The template for Quality Index Method is given in Appendix I. It was understood that in the Quality Index Method (QIM) that the demerit scores for all quality attributes increased with storage time in different storage condition. This was indeed observed but to a different extent for different quality attributes. Figure 5.1 shows the changes of quality scores of squid sample at

different storage temperature such as-18±2°C, 0±2°C, 4±2°C, and 30±2°C.The first evaluation of sensory changes was carried out at day zero.

Individual scores for each sensory parameter showed a similar increasing trend for samples stored under all treatments. Quality scores of squid sample on initial hour of storage at 30±2°C was 0, after 6 hours it was 8.125±0.13, then after 12 hours at the same storage conditions, quality scores were reached highest of approximately15.63±0.5 and the sample was rejected after 12 hour on the basis of sensory quality Fig.5.2. Changes of quality scores of squid sample stored at 4±2°Cin 2nd day of storage shows 4±0.7, and the demerit points gradually increased during storage and on 10th day quality scores of sample reached the maximum demerits point 16 Fig.5.1.The shelf life of samples stored at 4±2°Cwas 8-9 days. Quality scores of squid sample on 2nd of storage at 0±2°C reached to 2.75±0.46, and on 8th day at the same storage temperature the score was 8.75±0.707 and at 12th day of storage the quality score reaches the maximum demerit point 16. Samples were not evaluated for sensory beyond day 12 of storage due to strong off-odours and flabby texture leading to unacceptability.

The quality scores of organoleptic evaluation of Indian squid were given zero as an excellent quality. After that there was a high variability in the scores for skin colour, texture and odour in all storage conditions. The scores for skin mucus and odour increased consistently throughout the storage time, from approximately 0 to almost the maximum score. At the beginning of the storage time when the squid was very fresh, the odour was described as fresh seaweed then the odour became neutral. During the later stages, the odour was described as intense metallic, fishy odour. Freshly caught fish contains low levels of volatile compounds, which contributed to the fresh like odours and

2,6 nonadienal has been reported to be responsible for the seaweed like odour at could a low odour threshold (0.001 ppb)(Olafsdottir & Fleurence, 1997). The sour and rotten odour may have originated from short chain fatty acids, alcohols, sulphur compounds and amines generated by microbial activity (Whitifield, 2003). The scores for the appearance and colour increase constantly throughout the storage time in different storage temperature from approximately 0–2 has been characterized to have a very bright, well defined pigments of different sizes and colours to dull, without shine, purplish in the central axis of the body at the final stage. The study shows that the skin and muscle colour could be used for primary quality evaluation. It also reported that the decrease in sensory quality is related to a decrease in skin sheen and reddish brown colour, accompanied by a muscle stain formation (Ke, et al., 1984., Lakshmananan, et al., 1993).

The flavor and texture of stored raw squid at different storage temperature deteriorated at a similar and faster rate than other quality attributes.The scores for the quality attributes of flesh texture increase rather constantly throughout the storage time in all storage conditions from 0-2 even though the scores varied somewhat with storage time. The texture was described as firm, consistent when the squid was fresh which is turned to soft and finally flaccid and flabby. The soft flabby appearance increased with storage time in all treatments. However, at 4±2°C and 30±2°C the flavor and texture of raw squid deteriorated slightly faster than that of squid stored at -18±2°C and 0±2°C. Endogenous proteases are known to play an important role in the softening of cephalopod tissues (Hurtado, et al., 1999; Hatate, et al., 2000). Highly active cathepsins and other acid proteases in the digestive gland are rapidly released to the extracellular medium, causing degradation of muscle proteins and softening of the flesh; alkaline protease activity also

increases shortly after death, owing to the breakdown of zymogene vesicles (Le Bihan, et al., 2006). High proteolytic activity produced an increase in the level of non-protein nitrogenous compounds, hence favouring proliferation of the spoilage flora and rapid decomposition. During the later stages of storage, the skin lost its elasticity, becoming rather loosely attached to the underlying tissues, and the tentacles could be more easily removed.

Based on sensory analysis (odour, texture), the shelf life of squid samples stored at 0±2°C was 10-11 days and shows slightly better sensory characteristics than samples stored at 4±2°C. Further, the squid remained in acceptable condition for 10 h at 30ºC and 8 days at 4±2°C and 10 days at 0±2°C.

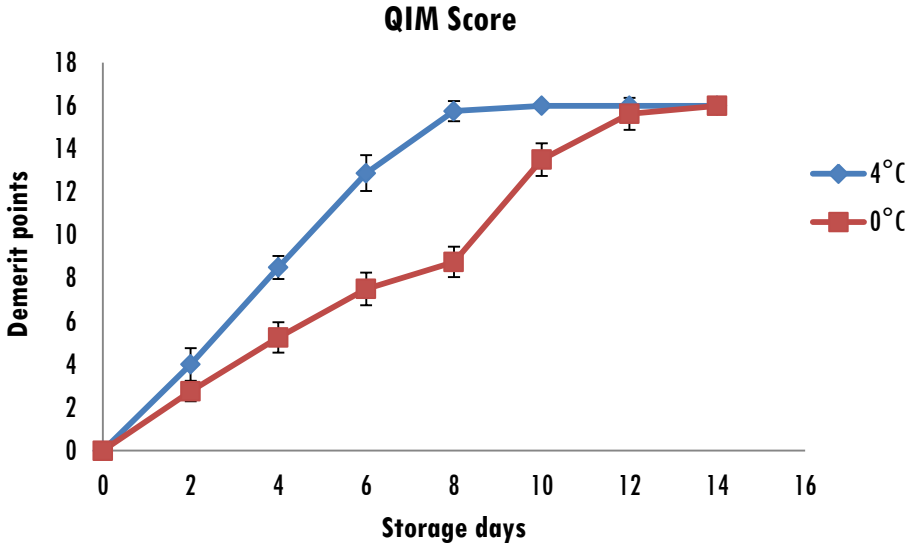


Fig. 5.1 Attribution of demerit points for sensory quality during storage days at 0±2°C and 4±2°C. Vertical bars show standard error.

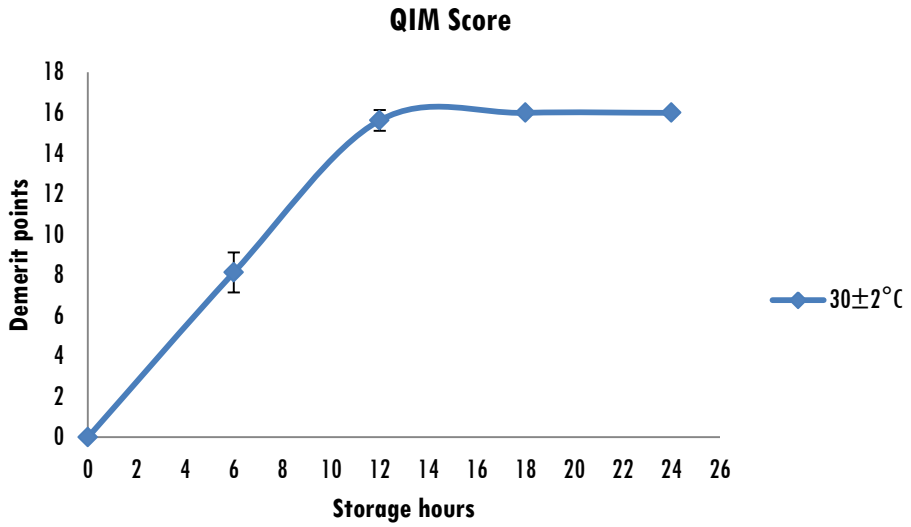


Fig. 5.2 Attribution of demerit points for sensory quality during storage days at 30±2°C. Vertical bars show standard error.

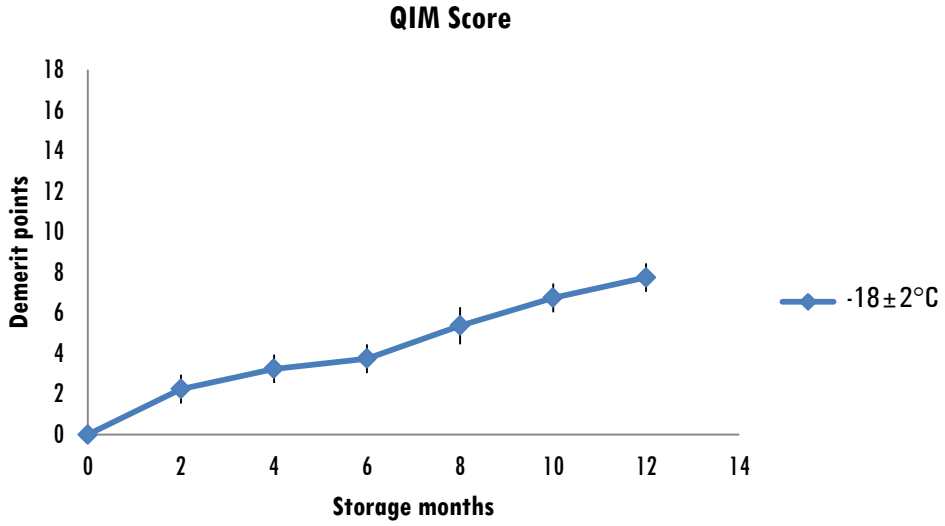


Fig. 5.3 Attribution of demerit points for sensory quality during storage days at -18±2°C. Vertical bars show standard error.

5.4.2 Chemical Analysis

5.4.2.1 Biogenic amines

Putrescine

Table 5.1 show the production of biogenic amines in squid muscles stored at different storage temperature. The formation of seven biogenic amines namely, putrescine (PUT), cadaverine (CAD), spermidine (SPE), spermine (SPER), histamine (HIS), tyramine (TYR) and agmatine (AGM) were followed during the study. The analysis of variance of biogenic amines especially PUT, CAD, HIS, AGM, and TYR showed significant difference between treatments ($p < 0.05$) and storage days ($p < 0.05$) and they increased with the storage period.

Among the biogenic amines putrescine,cadaverine, and histamine were not detected in squid muscle in the beginning of storage (Table 5.1) which increased rapidly at the stage of advanced decomposition. In samples stored at temperatures 0±2°C and 4±2°C the formation of putrescine increased 2nd day onwards and was reaches to maximum of 146.1 mg/ kg, and 183.2 mg/kg respectively. In samples stored at relatively low temperatures (-18±2°C) the formation of putrescine was not detected after two month but started appearing around 4th month of storage and then it reaches to 34.12 mg/kg at the end of the storage day (14th month). These values were significantly different ($p < 0.05$) compared to the concentration levels of samples stored at higher temperatures. The sample stored at room temperature (30±2°C) the putrescine content reaches to 145.3 mg/kg after 24 hours. The putrescine content of squid samples stored at higher temperature shows significantly ($P < 0.05$) higher values compared to other samples. Putrescine level increased gradually and

reaches the highest in squid sample in all storage condition at the end of the storage day except the sample at -18±2°C.

Similar results were reported for different squid species *Loligo subulata* (Prester, et al., 2010; Ozogul, 2008) *Todarodes pacificus (*Yamanaka, 1987). Paarup, et al., (2002) suggested that rapid putrescine formation in decomposed squid is owed to early bacterial conversion of agmatine. It also reported that putrescine has no adverse health effects but high putrescine levels in decomposed squid muscle could potentiate the toxicity of histamine and tyramine (Lehane, 2000).

Cadaverine

In the beginning of the storage the samples did not contain cadaverine (Fig. 5.1). Cadaverine formation was comparatively low in samples, which were stored at low temperature storage (-18±2°C) and the concentration of cadaverine remained below 13.4±0.32 mg/kg and these levels were significantly different ($p < 0.05$) compared to the samples stored at higher temperature. This was in accordance with earlier data reported by Slerm and Beyermann (1984). The highest amount of cadaverine was formed in samples stored at the chilled temperature (4±2°C). In this temperature the formation of cadaverine increased from 2nd day of storage (5.2±0.15 mg/kg). After 14 days of storage the concentration was 102.2±0.20 mg/kg, which was significantly higher ($p < 0.05$) level compared to samples in other storage temperature. However sample stored in 30°C the concentration of cadaverine reached a level of 100.4±0.33mg/kg in 24 hours of storage. In temperatures 0±2°C, the formation of cadaverine (3.3±0.29 mg/kg) was slow until 4 days of storage and after 14 days of storage the concentrations were between 3.3±0.29 and 92.4±0.32 mg/kg. It is interesting to note that the cadaverine production at

30±2°C could be reached in 4±2°C and 0±2°C, only after 14th day of storage. There were a significant ($p < 0.05$) differences in concentration of cadaverine in these storage conditions.Cadaverine increased at the onset of fish spoilage and may be taken as a chemical indicator of fish quality (Shakila, 2002).

Agmatine

Agmatine was detected in all samples at the beginning of storage and the values of agmatine showed an increasing trend similar to that of other biogenic amines except spermine and spermidine, which was more obvious in samples stored under 30±2°C.The squid samples stored at relatively low temperatures (-18±2°C) showed significantly different ($p < 0.05$) agmatine content (38.8±0.20 mg/kg in 14 months) compared to other sample stores at higher temperature. Agmatine concentration showed an increasing trend from 0 day onwards and reached a maximum on the 12thday at 0±2°C and thereafter a decline was observed. A similar trend was also observed during storage at 4±2°C storage temperature. Yamanaka, et al., (1987) indicated the possibility of agmatine as an index of freshness since agmatine concentration increased during the early stage of fish spoilage but decreased in later stages. Similar results were observed in this study also.

As storage time progressed, putrescine,cadaverine and agmatine became the dominant amines in the squid muscle.

Histamine

Histamine is considered to be the most important indicator of fish freshness because of its toxic effects (histamine intoxication). It is the only amine with a food safety concern and with regulatory limit of 100mg/kg (Commission Regulation, 2005). In the present experiment levels of histamine remained low in all temperature condition at the end of storage day, well

below the upper allowable limit for histamine 100 mg/ kg in scombroid fish. In squid sample stored at 30±2°C, no histamine was detected at 0 hr and a relatively low level of 2.21 mg/kg was detected after 24 hours storage. However this level is much higher than that of the histamine content in squid sample stored at other different storage temperature. Because of the low levels of histamine detected in the present experiment make such intoxication problems in squid irrelevant. The production of histamine is negligible compared to the scombroid fishes, because of the low level of amino acid histidine in squids (Alia, et al., 1992). This is in confirmation with the report of Frank, et al., 1985; Taylor, 1986; Ozogul, et al., 2002.

Tyramine

Tyramine was detected in all samples but at very low levels and the values showed an increasing trend, which was more obvious in samples stored at 4±2°C. Values of tyramine ranged from 1.3 to 2.9 mg/kg for the different temperature storage throughout the entire storage period. In samples stored at temperatures -18±2°C the formation of tyramine was consistent and at end of storage the tyramine concentration found 1.73 mg/kg which was significantly lower compared to the other samples. Even at 30±2°C, not much variation was noted and the level 2.4 mg/kg was reached in 24 hour of storage.

Other biogenic amines

No specific increasing or decreasing trend was observed for the rest of the biogenic amine. Spermine and spermidine were detected in all squid samples in the study, the level of spermidine remained low throughout the storage period in all temperature treatment but the level of spermine comparatively higher throughout the storage period in all treatment, which was expected since these two BAs are natural constituents in squid. Spermidine

and Spermine, which are naturally found in foods (Jamilah Bakar, 2010), were identified in all the samples studied without much changes. Spermidine concentrations in the samples for all temperatures were lower than that of spermine.

In general, the study shows histamine, putresciene, and cadverine were not detected in fresh squid sample, but were formed during storage and the levels significantly increased ($p < 0.05$) during storage. The agmatine and tyramine was detected in all samples at the beginning of storage. The level of concentration of spermine and spermidine did not much change during the storagein all samples.The toxicological level of biogenic amine depends on the individual characteristics and the presence of other amines (Brink, 1990; Halasz, 1994). Samples stored at -18±2°C had lower concentrations of the biogenic amines as compared to those stored at higher temperature. The quantity of amines formed in the squid at 30±2°C was quite higher than other temperature.Variation in the formation of different amines at different temperatures was probably due to the effect of temperature on the amine forming bacteria. The growth and activity of the amine forming bacteria varied at different temperatures and according to the availability of the amino acid substrates (Yamanaka, et al., 1986, Okuzumi, et al., 1990). The concentration of cadaverine and putrescine increased remarkably at the end of the storage days in all treatment and also at this stage, the sensory quality fell and microbial count increased. Cadaverine increased at the onset of fish spoilage and may be taken as a chemical indicator of fish quality (Jeya Shakila, 2002). Frank, et al., (1985) reported that the biogenic amine formation in fish is due to the activity of mesophilic more than psychrophilic bacteria. Valle, et al., (1996) found that when herring (*Clupeaharengus*) was inedible, putrescine and cadaverine contents of the herring stored at 0^0C were at 10.1 and 23

mg/kg, respectively. Middlebrooks, et al., (1988) also reported that the levels of histamine, cadaverine, and putrescine and the time and temperature of decomposition in Spanish mackerel (*Scomberomorus maculatus*) showed a strong correlation

Table 5.1 Mean concentrations of biogenic amines (mg/Kg) in *L.duvauceli* at different storage temperature (n = 3)

a) 4±2°C

4±2°C	mg/Kg						
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPER**
0th	ND	ND	ND	4.1±0.15^{a}	1.3±0.10^{a}	5.6±0.13^{a}	233.3±0.58^{a}
2nd	8.5±0.41^{a}	5.2±0.15^{a}	ND	19.4±0.32^{b}	1.5±0.08^{ab}	4.2±0.08^{bcd}	257.4±0.55^{g}
4th	32.4±0.32^{b}	16.3±0.25^{b}	1.1±0.12^{a}	56.4±0.32^{c}	1.8±0.12^{bcd}	3.3±0.29^{bcdef}	234.2±1.26^{a}
6th	78.4±0.36^{c}	43.3±0.26^{c}	1.4±0.13^{ab}	102.3±0.31^{d}	2.2±0.10^{d}	2.5±0.41^{ef}	242.0±1.05^{b}
8th	99.3±0.26^{d}	71.4±0.32^{d}	1.5±0.05^{bcd}	140.7±0.64^{e}	2.5±0.06efg	4.1±0.11^{bcd}	236.2±0.72^{a}
10th	134.1±0.1^{e}	85.4±0.36^{e}	1.7±0.12^{cdef}	142.7±0.31^{f}	2.6±0.11fg	3.5±0.13^{bcdef}	254.0±1.00^{f}
12th	164.7±0.15^{f}	94.2±0.26^{f}	1.8±0.05^{ef}	140.2±0.15^{e}	2.7±0.17fg	3.9±0.09^{bcd}	248.1±1.10^{c}
14th	183.2±0.15^{g}	102.2±0.20^{g}	1.9±0.12^{f}	138.6±0.32^{g}	2.8±0.07g	2.5±0.10^{f}	245.7±0.58^{ce}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

b) 0±2°C

0±2°C	mg/Kg						
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPER**
0th	ND	ND	ND	4.1±0.15^{a}	1.4±0.10^{a}	5.3±0.40^{ab}	233.1±1.01^{a}
2nd	4.2±0.16^{h}	1.3±0.23^{h}	ND	12.2±0.21^{h}	1.5±0.07^{abc}	4.4±0.42^{bc}	236.0±1.05^{a}
4th	14.3±0.31^{i}	3.3±0.29^{i}	ND	29.2±0.15^{i}	1.7±0.10^{abc}	3.9±0.69^{bd}	241.7±0.64^{b}
6th	25.1±0.10^{j}	12.3±0.26^{j}	1.2±0.21^{ab}	95.4±0.36^{j}	1.8±0.09^{cd}	5.2±0.37^{abc}	251.0±0.95^{ef}
8th	57.4±0.36^{k}	34.6±0.49^{k}	1.4±0.11^{ab}	125.2±0.15^{k}	2.0±0.13^{cd}	4.4±0.43^{bc}	246.0±1.00^{c}
10th	72.3±0.26^{l}	51.5±0.42^{l}	1.4±0.07^{bc}	132.6±0.49^{l}	2.2±0.13^{def}	2.5±0.41^{f}	225.0±2.65^{d}
12th	105.4±0.32^{m}	68.2±0.21^{m}	1.5±0.11^{bcdef}	136.4±0.36^{m}	2.4±0.11^{ef}	3.2±0.31^{def}	247.2±0.76^{c}
14th	146.1±0.10^{n}	92.4±0.32^{n}	1.8±0.12^{def}	128.3±0.26^{n}	2.4±0.34^{ef}	3.6±0.53^{bcd}	247.0±1.00^{c}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

c) -18±2°C

-18±2°C	mg/Kg						
MONTHS	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPER**
0th	ND	ND	ND	4.1±0.15^{a}	1.3±0.10^{a}	5.13±0.56^{a}	233.1±0.01^{a}
2nd	ND	ND	ND	4.7±0.29^{a}	1.4±0.08^{a}	4.87±0.51^{ab}	235.0a±1.0^{ab}
4th	2.2±0.21^{a}	0.9±0.11^{a}	ND	6.1±0.15^{b}	1.4±0.11^{ab}	4.35±1.17^{ab}	236.1±1.01^{bc}
6th	4.5±0.47^{b}	2.2±0.15^{b}	1.2±0.17^{a}	6.2±0.18^{b}	1.5±0.11^{ab}	4.84±0.57^{ab}	239.7±1.42^{d}
8th	10.3±0.26^{c}	5.6±0.55^{c}	1.2±0.07^{a}	13.3±0.31^{c}	1.5±0.09^{abc}	3.03±0.50^{bc}	238.1±1.03^{cd}
10th	15.8±0.15^{d}	6.3±0.25^{c}	1.3±0.05^{a}	16.3±0.26^{d}	1.6±0.11^{abc}	3.16±1.01^{bc}	241.0±1.05^{d}
12th	26.2±0.20^{e}	8.3±0.26^{d}	1.3±0.11^{a}	25.4±0.32^{e}	1.7±0.10^{bc}	2.06±0.55^{c}	238.3±1.15^{cd}
14th	34.0±0.06^{f}	13.4±0.32^{e}	1.4±0.11^{a}	38.8±0.20^{f}	1.7±0.09^{c}	3.13±0.12^{bc}	253.7±0.58^{e}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

d) 30±2°C

30±2°C	mg/Kg						
TIME (hours)	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPER**
0	ND	ND	ND	4.1±0.12^{a}	1.5±0.12^{a}	5.6±0.13^{a}	233.1±1.01^{a}
6	24.4±0.32^{a}	16.8±0.12^{a}	ND	62.2±0.12^{b}	1.9±0.14^{ab}	3.9±0.07^{b}	227.3±0.58^{b}
12	93.1±0.15^{b}	66.4±0.32^{b}	1.5±0.10^{a}	128.2±0.12^{c}	2.2±0.15^{bc}	4.1±0.10^{b}	261.3±1.53^{c}
18	129.4±0.31^{c}	82.0±0.06^{c}	1.4±0.33^{a}	137.3±0.12^{d}	2.3±0.25^{bc}	3.7±0.06^{c}	234.1±1.10^{a}
24	145.2±0.11^{d}	100.4±0.33^{d}	2.2±0.16^{b}	143.4±0.12^{e}	2.4±0.38^{c}	3.9±0.10^{bc}	237.4±1.58^{d}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

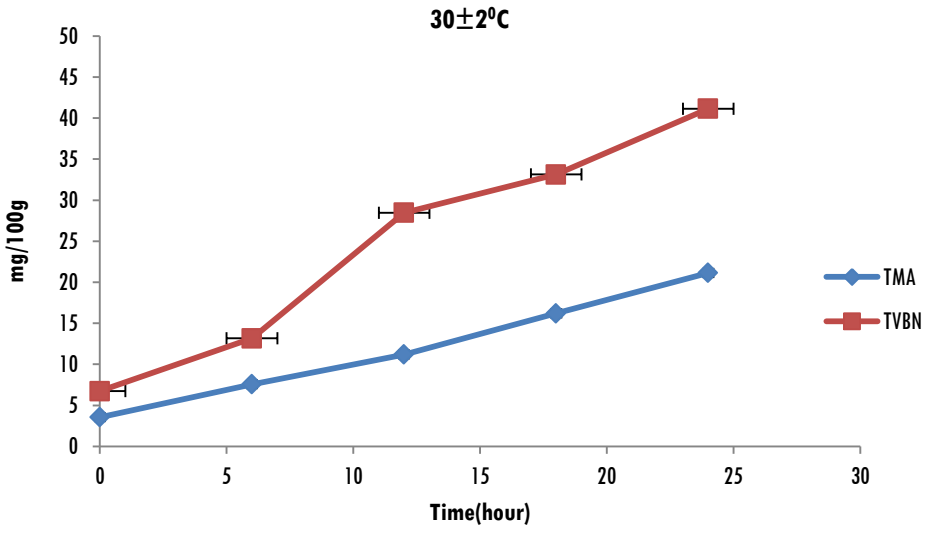
5.4.4.2 Trimethyl amine (TMA-N)

TMA-N is considered a valuable tool in the evaluation of the quality of fish stored in ice because of its rapid accumulation in muscle under refrigerated conditions (Kryzmien and Elias 1990).

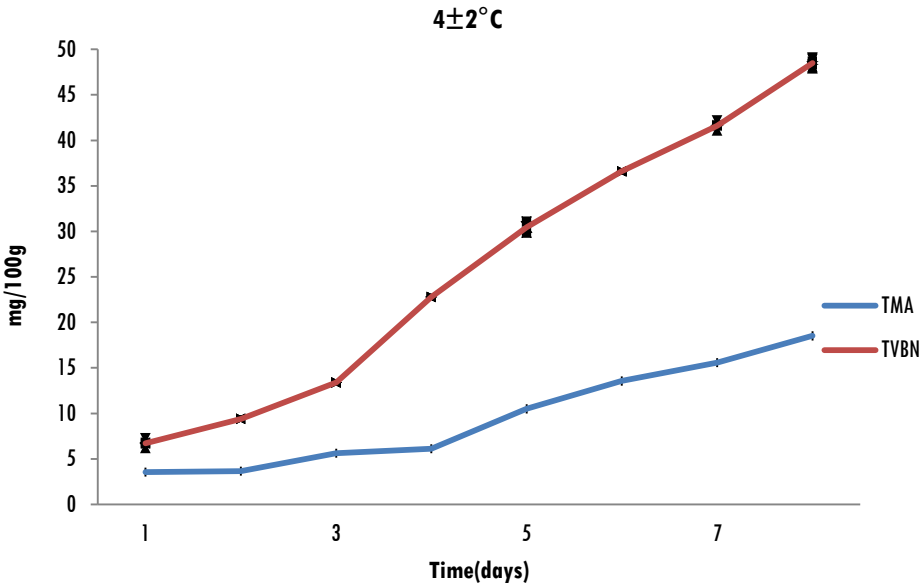
TMA-N resulting from bacterial reduction of osmoregulatory substances such as TMA-O is a pungent volatile amine often associated with the typical 'fishy' odour of spoiling seafood (Huss 1999). In this study, the production of TMA-N followed a pattern similar to TVB-N during different low temperature storage, but a significant increase was observed with storage time (P<0.05). The initial mean TMA-N content of squid muscle was 3.6±0.05 mg/ 100 g. The TMA-N content of the squid sample increased slightly during the first 2 days of chilled storage 4±2°C and 0±2°C (Table. 5.2) followed by a

marked increase in TMA-N content was observed until the 14th day of the storage in 4±2°C (18.5±0.03 mg/ 100 g) and in 0±2°C(14.5±0.25 mg/ 100 g) (p□0.05). The sample stored at 30±2°C, the TMA-N content reaches to 21.1±0.12 after 24 hours storage. During frozen storage (Table.5.2) the TMA production was relatively low reaching a value of first 5.9±0.04 mg/100g in 12 months of storage; probably due to inactivation of bacteria at that temperature. The limit of TMA at spoilage is reported to be 10mg/100g (EC, 1995) and the level reached on 12days, 8 days and, 12 hours respectively at 0±2°C,4±2°C, and 30±2°C. Both TVB-N and TMA-N are relatively constant in the mantle of fresh caught cephalopods (Ruız-Capillas et al., 2002). There are reports in molluscs indicating increase in both TVB-N and TMA-N after storage in ice (Civera et al., 1999; Murata &Sakaguchi, 1986). Civera et al. (1999) observed an exponential increase in TMA-N due to different storage conditions. The present results of TVB-N and TMA-N during the linear phase were similar to the initial results found by Albanese et al. (2005) for the cephalopods.

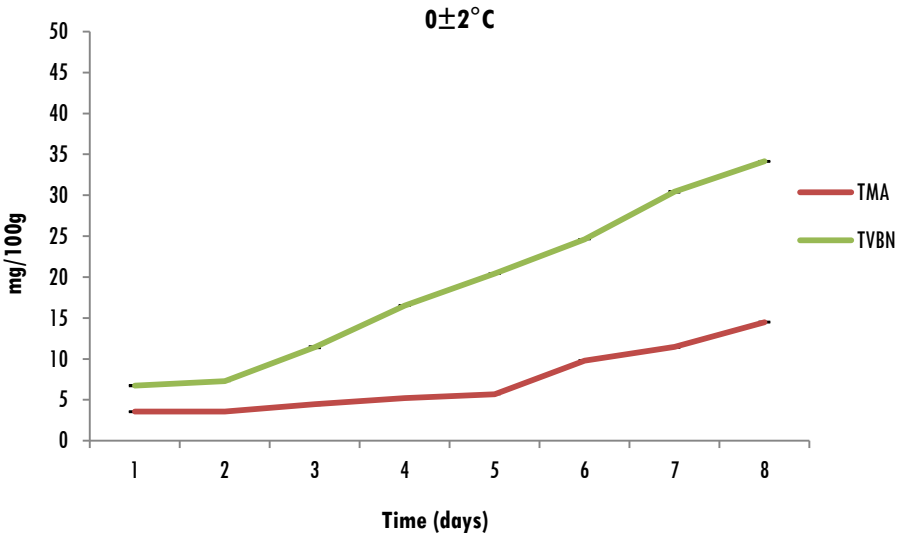
a.



b.


4±2°C
mg/100g
50
45
40
35
30
25
20
15
10
5
0
1
3
5
7
Time(days)
TMA
TVBN

c.


0±2°C
mg/100g
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
Time (days)
TMA
TVBN

d.

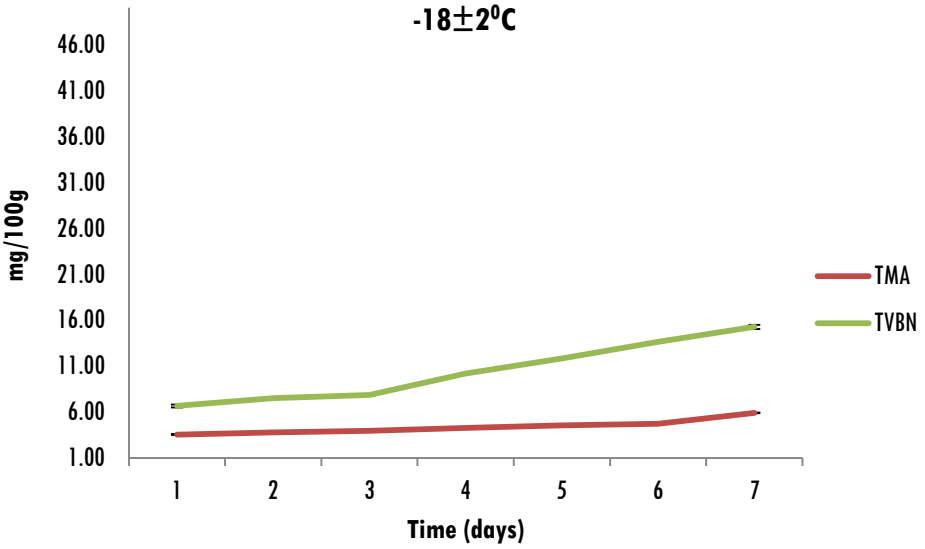


Fig. 5-4 (a-d) Changes in TMA and TVBN in *L.duvauceli* during storage at different storage temperature

Table 5.2 Mean concentrations of TMA (mg/100g) in *L.duvauceli* at different storage temperature (n = 3)

TMA(mg/100 g)			TMA(mg/100 g)		TMA(mg/100 g)	
Days	**4±2°C**	**0±2°C**	**Hour**	**30±2°C**	**Months**	**-18±2°C**
0th	3.6±0.04a	3.6±0.05a	0	3.6±0.05a	0	3.6±0.05a
2nd	3.7±0.05a	3.6±0.06a	6	7.6±0.06b	2	3.8±0.02b
4th	5.6±0.28d	4.5±0.03b	12	11.2±0.18c	4	3.9±0.04b
6th	6.1±0.11e	5.2±0.06c	18	16.2±0.18d	6	4.3±0.03c
8th	10.5±0.01g	5.7±0.08d	24	21.1±0.12e	8	4.5±0.01d
10th	13.6±0.05i	9.8±0.01f	-	-	10	4.7±0.03e
12th	15.6±0.06k	11.5±0.06h	-	-	12	5.9±0.04f
14th	18.5±0.03l	14.5±0.25j	-	-	14	7.1±0.17g

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

5.4.4.3 Total volatile base nitrogen (TVBN)

Determination of TVB-N, widely used to assess the freshness of fish, and related commodities correlates well with sensory changes during spoilage. TVB-N involves combined measurement of trimethylamine, dimethylamine,

ammonia and other compounds in relation to spoilage. At the beginning of storage, the TVB-N content in freshsquid was 6.7±0.07 mg/100 g. This initial level was probably due to the endogenous production of ammonia from the enzymatic degradation of protein, amino acids, and nucleotides immediately post-mortem. The pattern of change in TVB-N content in squid samples during different storage period is shown in table 5.2

TVB-N content increased with increasing storage time in all storage conditions (p□0.05). TVB-N content increased rapidly from 4th day onwards in 4±2°Cand 0±2°C storage. At the end of 4±2°C and 0±2°C storage, the mean TVB-N content in squid muscle were 48.5±0.15 and 34.1±0.05 mg/100 g. During storage at ambient temperature (30±2°C) the value, increased significantly reaching 41mg/100g in 24 hours (Table 5.3). At -18±2°C the TVBN production was much slower and a value of 15mg/100g was detected after 12 months of storage (Table 5.3). Similar results were found by Ruiz-Capillas, et al., (2002) for other cephalopod species. The limit for TVB-N content in edible fish has been reported to be 25-35 mg/100 g (EC, 2008., Woyewoda, et al., 1986). In this study, the TVB-N contents of squid muscle under 4±2°C and 0±2°C storage were exceeded the limit after 10 and 14 days of storage. Whereas the squid samples stored at room temperature was exceeded the limit after 18 hours of storage. The TVB-N content of squid muscle under frozen condition does not exceed the limit after 12 months. Thus, the low temperature storage is important in controlling TVB-N content in squid muscle. Generally speaking, TVB-N reflects only stages of advanced spoilage and is considered unreliable for the measurement of spoilage during the first 10 days of cod's ice storage as well as for several other species (Huss, 1999). According to Botta (1995), TVB-N values are not linearly related to the spoilage of the species stored in ice, and cannot be used to predict its life storage. However, TVB-N values do identify the later stages of spoilage and,

therefore, can be used as a routine/standard method to determine if chilled seafood is spoiled.

Table 5.3 Mean concentrations of TVBN (mg/100g) in *L.duvauceli* at different storage temperature (n = 3)

TVBN (mg/100 g)			TVBN (mg/100 g)		TVBN (mg/100 g)	
Days	**4±2°C**	**0±2°C**	**Hour**	**30±2°C**	**MONTHS**	**-18±2°C**
0th	6.7±0.07a	6.7±0.07a	0	6.7±0.07a	0	6.7±0.16a
2nd	9.4±0.01b	7.3±0.01c	6	13.2±0.06b	2	7.5±0.01b
4th	13.4±0.02d	11.4±0.11e	12	28.5±0.06c	4	7.9±0.06c
6th	22.8±0.01f	16.5±0.02g	18	33.1±0.12d	6	10.2±0.01d
8th	30.5±0.59j	20.4±0.03h	24	41.1±0.12e	8	11.8±0.03e
10th	36.6±0.02l	24.6±0.02i	-	-	10	13.6±0.03f
12th	41.6±0.04m	30.4±0.11j	-	-	12	15.3±0.23g
14th	48.5±0.15n	35.1±0.05k	-	-	14	17.3±0.06h

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

5.4.2.4 pH

The pH is commonly used as an indicator to understand the quality of meat which indirectly indicates the fish deterioration (Howgate, 2009). The normal pH of squid meat ranges from 6.25-6.40. The pH of the squid muscle increased with storage time in different storagetemperature(Table 5.4). The study shows squid muscle pH varied from 6 to 7.5. Researchers stated that postmortem pH of fish muscle can vary from 6.0 to 7 depending on season, species, and other factors (Simeonidou, et al., 1998; Church, 1998).The similar increasing trend has frequently been reported by different researchers (Magnusson, et al., 2009; Susanto, et al., 2011; Abelti, 2013.). Postmortem muscle pH value varies between 6 to 7 have been reported for several squid species, including *Loligo duvaucelii, Loligo vulgaris* (Eiji,Ohashi et al., 1991; Ozogul, 2008; Paarup, et al., 2002).

In the study pH of the muscle varied with storage time, it was slightly higher (14th day) in 4±2°C(7.47) storage than in 0±2°C (7.22), whereas lower

pH (6.66) obtained on -18±2°C, this is probably due to difference in storage temperature, and there were insignificant difference between days storage in all storage conditions. The pH of squid muscle in the 0 hour at 30±2°C was lower (pH 6.4) than after 24hours storage (pH 7.4) at same storage conditions. Similar patterns were obtained for other storage condition also. The increases in pH were associated with the formation of volatile bases derived from microbial action on fish muscle as evidenced by the increases in TVB, and TMA contents (Kyrana, 1997). The increase in pH at the end of the storage day at different temperature was associated with the state of rapid spoilage of the squid (Capillas and Moral 2005). The result was in conformity with the result from microbiological analysis. Slower the bacterial growth led to the lower pH of fish flesh (Okeyo, et al., 2009).

Table 5.4 Changes in pH in *L.duvauceli* at different storage temperature (n = 3)

pH			pH		pH	
Days	**4±2°C**	**0±2°C**	**Hour**	**30±2°C**	**Months**	**-18±2°C**
0th	6.25±0.22^{a}	6.3±0.6^{ab}	0	6.4±0.09^{a}	0	6.4±0.05^{a}
2nd	6.59±0.10^{bcd}	6.5±0.08^{abc}	6	6.5±0.06^{ab}	2	6.4±0.10^{ab}
4th	6.68±0.12^{cd}	6.5±0.10^{abc}	12	6.9±0.09^{bc}	4	6.4±0.11^{abcd}
6th	6.89±0.05^{de}	6.6±0.01^{bcd}	18	7.0±0.13^{cd}	6	6.5±0.06^{bcd}
8th	7.24±0.13^{fg}	6.8±0.08^{cde}	24	7.4±0.36^{d}	8	6.5±0.01^{abc}
10th	7.31±0.11^{fg}	6.8±0.05^{de}	-	-	10	6.6±0.05^{cde}
12th	7.37±0.10^{g}	7.0±0.16^{ef}	-	-	12	6.7±0.05^{de}
14th	7.47±0.08^{g}	7.3±0.12^{fg}	-	-	14	6.9±0.05^{e}

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

5.4.3 Bacteriology

5.4.3.1Total Plate Count (TPC)

In order to confirm the influence of microbial flora on formation of biogenic amine, microbiological study was carried out at different storage conditions. Table 5.5 shows the aerobic microbial flora in squid samples

stored at 0±2°Cand 4±2°C. Higher counts were observed at 4±2°C as compared to the counts at 0±2°C. At day 0, the initial total plate counts were 3.6±0.07log CFU/g. At day 14th the counts were increased to 6.4±0.04 and 7.5±0.04log CFU/g for samples stored at 0±2°C and 4±2°C, respectively. If 6 log CFU/g is taken as the TPC limit of acceptability (FAO, 1999), therefore the shelf-life of squid was approximately 10 and 12 days at 4±2°Cand 0±2°C, respectively. There were significant differences ($P < 0.05$) in total plate count of squid stored at 0±2°C and at 4±2°Cduring the storage days.

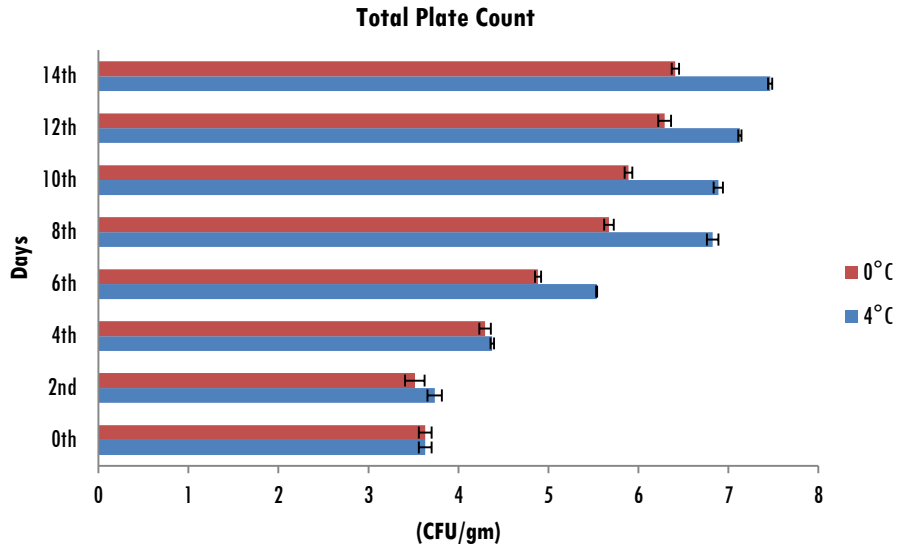
Table 5.5 shows the total plate counts in squid samples stored at 30±2°C and -18±2°C The initial plate count of the squid muscles was 3.6±0.07 log cfu/g and was increased gradually and reached 7.86±0.06log cfu/g after 24 h at 30±2°C and 5.5±0.14 cfu/g at -18±2°C after 14 months. Microbial counts are also known to vary with the water from where the squids are caught and the handling practices, and hence it cannot directly be a reliable index of quality. El Marrakchi, et al., (1990) also reported that the total viable counts in fish stored at ambient temperature exceeded the acceptable limit faster as compared to those kept on low temperature. Microbial quality of squid samples was in accordance with the biogenic amines and sensory evaluation.

Table 5.5 Changes in total plate count in *L.duvauceli* at different storage temperature (n = 3)

TPC log CFU/gm			TPC log CFU/gm		TPC log CFU/gm	
Days	**4±2°C**	**0±2°C**	**Hour**	**30±2°C**	**Months**	**-18±2°C**
0th	3.6±0.07ab	3.6±0.07ab	0	3.6±0.07a	0	3.6±0.07a
2nd	3.7±0.08b	3.5±0.11a	6	3.7±0.01b	2	3.7±0.03a
4th	4.4±0.02c	4.3±0.07c	12	6.6±0.02c	4	3.9±0.02a
6th	5.5±0.01e	4.9±0.03d	18	6.9±0.02d	6	4.2±0.10b
8th	6.8±0.06h	5.7±0.05e	24	7.5±0.06e	8	4.3±0.05b
10th	6.9±0.05h	5.9±0.04f	-	-	10	4.9±0.07c
12th	7.1±0.02i	6.3±0.07g	-	-	12	5.0±0.22c
14th	7.5±0.02j	6.4±0.04g	-	-	14	5.5±0.14d

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly ($P<0.05$)

a.



b.

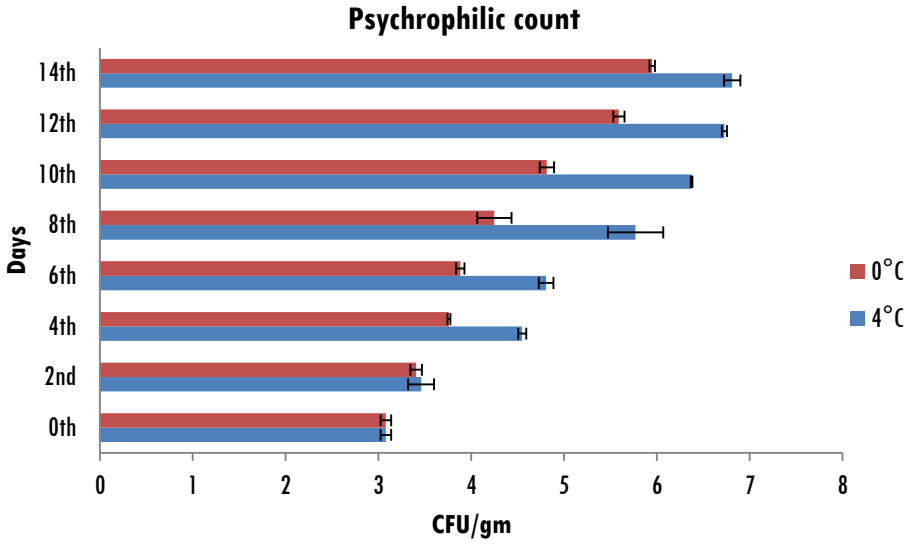


Fig. 5-5 (a-b) Changes in total plate count and psychrophilic count in *L.duvauceli* at different storage temperature

5.4.3.2 Psychrophilic count

The results in table 5.6 showed that the counts of psychrophilic bacteria were found to significantly increase ($p < 0.05$) with increases of storage time in squid samples at different storage temperatures. In the samples, the initial counts of psychrophilic bacteria were at 30±2°C, shows 3±0.02 log cfu/ g, and after 24 hours of storage at the same temperature reached levels of 4.7±0.07 log cfu/g. In samples stored at -18±2°C, the psychrophilic counts were of lower than the samples stored at other temperature. The counts of the psychrophilic bacteria stored at 0±2°C were lower than samples at 4±2°C, reaching 6.4±0.04 log cfu/g (0±2°C) and 6.8±0.04 log cfu/g (4±2°C), clearly indicating the significance of chilling / frozen storage of squid. The results are in agreement with Gelman, et al., (2001). Frank, et al., (1985), stated that the biogenic amine production is strongly related to the activity of mesophilic bacteria than psychrotrophic bacteria. The best correlations among bacteria and amines were found.

Table 5.6 Changes in psychrophilic count in *L.duvauceli* at different storage temperature (n = 3)

PC log cfu/gm			PC log cfu/gm		PC log cfu/gm	
Days	**4±2°C**	**0±2°C**	**Hour**	**30±2°C**	**Months**	**-18±2°C**
0th	3.1±0.06a	3.1±0.06a	0	3.1±0.02a	0	3.1±0.06a
2nd	3.5±0.14b	3.4±0.06b	6	3.3±0.01a	2	3.3±0.04ab
4th	4.5±0.04e	3.8±0.02c	12	3.9±0.03b	4	3.5±0.04b
6th	4.8±0.08e	3.9±0.05c	18	4.6±0.30c	6	4.2±0.07c
8th	5.8±0.30fg	4.3±0.18d	24	4.7±0.07c	8	4.2±0.02c
10th	6.4±0.01h	4.8±0.08e	-	-	10	4.6±0.28d
12th	6.7±0.03i	5.6±0.06f	-	-	12	4.8±0.03d
14th	6.8±0.09i	5.9±0.03g	-	-	14	5.3±0.13e

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly ($P<0.05$)

5.5 Conclusions

It is known that squid has a short shelf-life once caught. Storage in low temperatures considerably delays spoilage occurrence and extends the shelf-life of squid. In this work the Indian squid stored at different storage conditions via;-18±2°C, 0±2°C, 4±2°C and 30±2°C, revealed that with increase in storage period decompositions occurred by biochemical and bacteriological interaction. Storage temperatures directly effect on shelf-life of squid, where the higher storage temperature caused rapid spoilage than the lower storage temperatures. The storage temperature of -18±2°C and 0±2°C prevent the growth of bacteria which slowed down the bacterial proliferation and reduced the decomposition in squid samples and reduce the production of biogenic amines, while storage temperatures of 30±2°C allowed the bacterial to proliferate faster which reduced the shelf-life of stored squid. Therefore, it is suggested to chill squid rapidly after catch and use low storage temperatures (≤4°C) to extend the shelf-life of squid. For the insurance squid quality, the chemical indicator such as the concentration of putrescine, cadverine, agmatine, histamine and tyramine shows a better correlation with the organoleptical and microbiological indicator to determine the quality and freshness of squid and to detect the squid spoilage.

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Chapter

6

EFFECT OF DELAYED ICING ON BIOGENIC AMINES FORMATION IN INDIAN SQUID (*Loligo duvauceli*)

Contents

6.1Introduction

Temperature is one of the key factors affecting the quality of raw fresh fish during handling and storage. In general, fishing operations are carried out overnight and stored is at ambient temperature until unloaded at the landing centre. Such delaying of catch for long period may cause spoilage organisms to multiply, an increase of 1000 times (three log scales) over the assumed initial level of 10/g or cm^2, reaching to a level of 10000/cm^2 at fish surfaces or 10000/g in the gut (FAO, 2004). Low temperature storage or chilling of squid as quickly as possible after catch are commonly recommended methods for controlling the growth of spoilage organisms and to maintain the freshness of squid (FDA, 2011). Freshness is the most important criterion when assessing squid quality (Connell, 1975). Biological, chemical and organoleptic evaluation has been used to judge freshness of squid during handling, processing and storage (Koutsoumanis et al., 2002). Quality of squid refers to

the aesthetic appearance or degree of spoilage which the squid has undergone after catch. The methods for the evaluation of squid quality are based on autolytic changes, development of microbial growth and sensory attributes (Huss et al., 1992). Biochemical methods are related to nucleotide metabolism, production of trimetylamine (TMA), hypoxanthine (Hx), and total volatile basic nitrogen (TVB-N) (Hasegawa, 1987). Analyzing biogenic amines have also been used to evaluate fish quality. Biogenic amines are significant not only due to their possible toxicity, but also can be used as indicators for quality of freshness or spoilage of foods (Silla Santos, 1996). Biogenic amines are produced at very low levels in fresh fish and their formation is related to bacterial spoilage (Ozogul&Ozogul, 2006). Amines such as cadaverine and putrescine are very important in fish and fish products, since they have been shown to potentiate the toxicity of histamine (Shalaby, 1996). Though biogenic amines in small concentrations are reported to be essential for many physiological functions (Bardocz et al., 1995; Eliassen, et al., 2002), ingestion of large amounts may result in health problems (Eerola, et al., 1998). Toxicity of amines strongly depends on the individual efficiency of detoxication (Shalaby, 1996).

The effects of temperature abuse on biogenic amines formation in different fish species have been studied extensively. Temperature related biogenic amine production during storage have been reported by many authors in different fish species (Kim et al.,2009; Visciano et al.,2007; Guizani et al.,2005; Du,et al., 2002; Kim et al.,2001; Wei et al.,1990). There are scientific studies providing information on quality changes of different species of squid after catch and during storage (Paarup, et al., 2002; Lapa-Guimaraes, Byun et al., 2000; Ohashi., 1991; Langille& Gill., 1984). In contrast, information on the production of biogenic amine in squid relation to storage temperature is

relatively scanty. Once landed, squid passes through different channels, with or without icing. High ambient temperatures and long pre-icing periods may accelerate the deterioration of squid quality. In this background, the present study was undertaken to determine the influence of delayed icing on the formation of biogenic amine in squid in relation to general quality attributes namely autolytic changes, development of microbial growth and sensory quality.

6.2 Review of Literature

Delayed icing is a common practice under actual commercial conditions due to various reasons. In some cases proper icing may not be done even at the landing centers but only at the processing plants or at the premises of fish traders. Icing of fish immediately after catch may not be practical in small boats because of limited facilities and non availability of ice on board. In the previous delayed icing study, fresh water prawn was investigated under various storage conditions. *Macrobrachium rosenbergii and Penaeusmonodon* species were found in organoleptically acceptable condition for 6 to 7 days and the delayed icing considerably shortened the shelf life of fresh water prawn (Rahman et al., 2001a; 2001b). Masoud et al (2008) reported the shelf-life of rainbow trout stored in ice immediately after catch was approximately 9–11 days, while delay in icing for 4 and 8 h shortened the shelf-life was around 5–7 and 1–3 days, respectively. Krishnakumar et al. (1985) found that oil sardine un-iced on board and later iced after landing was least preferred compared with that iced or preserved in chilled sea water immediately after catch . Curran et al., (1980) studied the shelf life of gold lined sea bream at 0°C (in ice) and at 10 °C and established that rate of spoilage at 10 °C is approximately five times more than at 0°C. Most of the bacterial contamination found on the landed fish appears to be related to the handling practice of the fish and bacterial growth during storage (Elinor et al., 1985).

According to Nair et al. (1974), chilling of fish before the complete resolution of rigor was not found to affect the quality of fish to any greater extent during subsequent storage, but further delay affects the shelf life. Jeyasekaran et al., (2006) reported immediate icing extended the shelf life of barracuda by 6 days. Ramachandran et al., (1990) studied theeffect of delayed icing on the microbiological quality of hilsa toil. Akankwasa (1998) reported that delayed icing considerably shortened the shelf life of fresh water Arctic charr (*Salvelinu salpinus*). Bhattacharya (1990) and Bandyopadhay (1986) reported the shelf-life of *clarias batrachus and catla catla* respectively under various storage conditions. Rubbi et al., (1985) also studied the shelf-life of six freshwater fish species in different storage temperatures. Seyed et al., (2013) studied the effect of delayed icing in common carp muscle. Mani Maran (2014) reported that delayed icing shortens the shelf life of octopus by 4 days, if held for 6h at ambient temperature.The available information suggests that the effect of delayed icing exerts serious threats on quality of fish and shellfish (Dawood et al., 1986). The decomposition of fish protein leads to the formation of peptides and amino acids, which are susceptible to further degradation, resulting in the formation of biogenic amines (BAs), which can be widely distributed in proteinaceous foods (Krizek, 2004).

6.3 Materials and Methods

The samples drawn daily and were analyzed for sensory, chemical and microbiology indices. The analyses of the above said parameters were conducted as described in Chapter 4.

6.3.1 Sample preparation

Fresh squid (*Loligo duvauceli*) sample weighing from 50 to 65 g were collected from a Thoppumpadi fish landing centre, Kochi, India and divided in to five lots containing 30 squids in each. One lot was immediately iced and the rest of four were brought to the laboratory and placed in trays at ambient temperature (30 ± 1^0C) and iced after 1, 2, 4 and 8 hours respectively in ice boxes with outlet for water drainage. The samples were chill stored for 14 days with 1:3 squid to ice ratio and the melted ice was replaced daily. At each sampling time, four squids were randomly selected and removed from the ice in triplicate and then the sensory attributes of squid determined. The experimental squid ascetically skinned, gutted, minced and homogenized for chemical biogenic amines, TMA, TVN, pH and microbiological analysis as per the method described under chapter 4. The sensory evaluation was parallelly done to confirm the freshness and acceptability.

6.3.2 Statistical analysis

The mean values and standard deviations of the experimental data from triplicates were calculated by Microsoft Office Excel 2003. Statistical analysis was performed in IBM SPSS Statistics version 20 and data reported as mean± standard deviation Multivariate ANOVA at 5% level of significance was performed to compare the treatment means. Turkey's multiple comparison test was used for post-hoc analysis.

6.4 Result and Discussion

6.4.1 Sensory analysis

The freshness evaluations of squid were performed using Quality Index method for shortfin squid (Vaz-Pires & Seixas, 2006). During the storage

period, squid showed gradual and consistent changes for all the parameters of sensory evaluation and reaching a total score of 16 demerits points. The changes in the sensory characteristics (skin appearance, odour, mucus, flesh texture, eye appearance and shape, mouth odour and appearance) of squid ice stored after delay icing for various time intervals are given in figure 6-1. The freshness are judged by the panel of experts on the basis of the organoleptic characteristics such as general appearance of the skin color, odor, texture of muscle, overall appearance, mouth region, ocular tissue and eye color were evaluated. (Sensory table; Appendix-1) The samples stored in ice immediately after catch onboard were organoleptically acceptable for 12 days while delayed icing of 1, 2, 4 and 8 hours shortened the shelf life to 8, 6, 4 and 2 days respectively. The rejection (demerit score 16) was occurring mainly due to the presence of unpleasant ammoniacal odour and flabby texture. According to Paulo Vaz-Pires (2006) reported that the shelf life, as measured by sensory attributes had to be 9 days in ice for squid.

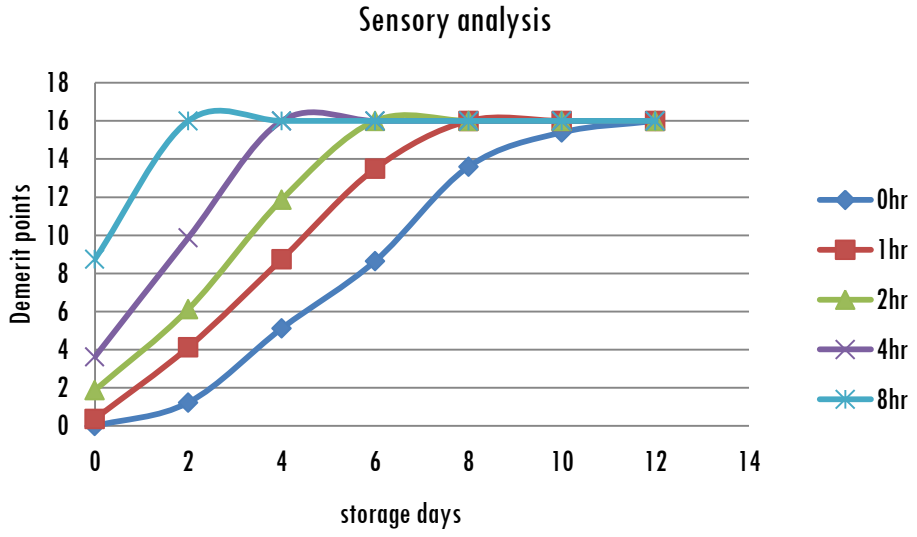


Fig: 6.1 Effect of delayed icing on sensory quality

6.4.2 Chemical Analysis

6.4.2.1Biogenic amine

In the present study, seven biogenic amines were studied namely, putrescine, cadaverine, tyramine, spermidine, agmatine, spermine and histamine.All the biogenic amines except PUT and HIS were present in trace amounts in fresh squid. The amines were increased rapidly in all lots except spermine and spermidine.

As presented in Fig. 6-2, Putrescine was found to be the second dominant biogenic amine shows significant changes in ice storage. Compared to other biogenic amine putrescine was not detected in the initial day in the samples immediately iced and iced after 1hour. In the samples immediately iced and iced after 1hour putrescine was detected 3.46 and 3.56 mg/kg on 2nd day and it was significantly increased to 110.5 and 111.2 mg/kg during the storage period of 12 days. A distinct increase was noticed in PUT, in all delayed iced samples during storage and a value of 203.94 mg/kg was recorded in the squid sample iced after 8 hours on day 12 of storage.Valle, et al., and (1996) found that when the fish became inedible the putrescine contents of herring stored at 0^{o}C was 110 mg/kg. In the present study, it was clear that delayed iced squid samples had a significant effect on putrescine production throughout the entire storage period in ice. The higher levels of putrescine in sample lots iced after 8 hours as compared to immediately iced samples may be related to higher counts of spoilageorganisms in delayed iced samples. Recently, it was reported in carps that putrescine values lower than 10 mg/kg for good quality carp meat, values between 10 and 20 mg/kg for acceptable quality and values over 20 mg/kg for poor quality carp meat based on sensory scores (Krizek et al., 2002).

The change in cadaverine has been indicated in Fig 6-2. Cadaverine was found to be the third dominant aminein all lots, increasing from an initial level of 1.08 mg/kg to 60.1 mg/kg in immediately iced samples during 12 days of ice storage. Low values of cadaverine recorded during the initial stages of storage, are may be associated with the good quality or freshness of squid which increased rapidly in all lots during storage. In delayed icing 8 hours, the CAD concentrations was 16.5 in the initial day and reaching a maximum of 146.87mg/kg at day 12 (Fig. 6-2 (e)). The rate of increase in the concentration of cadaverine was found greater in and after 6 days of storage in all lots. Lakshmanan, (2002) observed emperor fish and of shrimp that the bacteria that produce cadaverine and putrescine survive and multiply rapidly between 9 and 12 days and contribute to the formation of amines after the ice storage. Yamanaka et al., (1989) have suggested that cadaverine (upper acceptable limit of 10 mg/kg) may be used as an indicator of freshness in salmonoids. Cadaverine originates from the decarboxylation of lysine and has been associated with the bacteria *Enterobacteriaceae* (Halasz, 1994). Cadaverine and putrescine have both beensuggested as spoilage indicators of squid (Krizek et al. 2002; Yamanaka et al. 1987). Dawood et al., (1988) suggested using the levels of these two amines could be used to assess freshness of chill-stored rainbow trout. A good correlation was found between sensory evaluation and levels of putrescine and cadaverine in this storage and is in accordance with the findings in skipjack tuna (Sims et al., 1992).

Agmatine showed significant changes in ice storage Fig. 6-2. In the present study the agmatine value reached over 130.5 mg kg^{-1} on day 12 for samples iced immediately, while it was 142.2 and 152.4 mg kg^{-1} after 12 days for the samples with 2 and 4 h delays. The maximum value for agmatine was observed on day 12 for 8 h delayed storage (166.4 mg kg−1). The same pattern was seen in all lots and the value of sample with 8 h delay was higher than for

0 and 4 h delay.The very early onset of agmatine production indicates an autolytic mechanism, but the fact that it is produced by decarboxylation of arginine suggests the involvement of bacterial decarboxylase activity. But the rate of production of agmatine was not consistent throughout ice storage, after a stable initial period. At the end of the storage period the rate of production of agmatine contents was gradually decreased in all the treatments. This peaking behavior has also been described during the storage of pelagic fish (Veciana-Nogues, et al., 1997; Yamanaka, et al., 1989). The peak profile may be due to the fact that agmatine is an intermediate metabolite in the putrescine formation pathway from arginine (Veciana-Nogues, et al., 1995). Therefore, the use of agmatine does not seem to be appropriate to evaluate squid freshness, because it did not show a regular pattern of change throughout ice storage.This amine seems to be an excellent freshness index in squid, which is in agreement with Yamanaka et al. (1987).

As illustrated in Fig. 6-2, tyramine was found in low concentration (less than 4mg/kg) in all the lots during ice storage, but shows an increasing pattern. Tyramine appeared from initial day onwards (1.7 mg/kg) and the concentration was increased to 1.83 mg/kg on day 12 for immediately iced storage.The maximum value for tyramine was observed on day 12 for 8 h delayed storage (3.15 mg/kg).Tyramine was a minor amine from a quantitative point of view, but its occurrence in squid should not be undervalued, because it is also related to fish spoilage, as also reported in other works(Krizek et al. 2004).Krizek et al. (2004) and Yen andHsieh (1991) reported low contents (<10 mg/kg) of tyramine in fresh water carp and canned tuna. However, the maximum recommended level of tyramine in food should be in the range of 100–800 mg/kg (Brink, 1990).

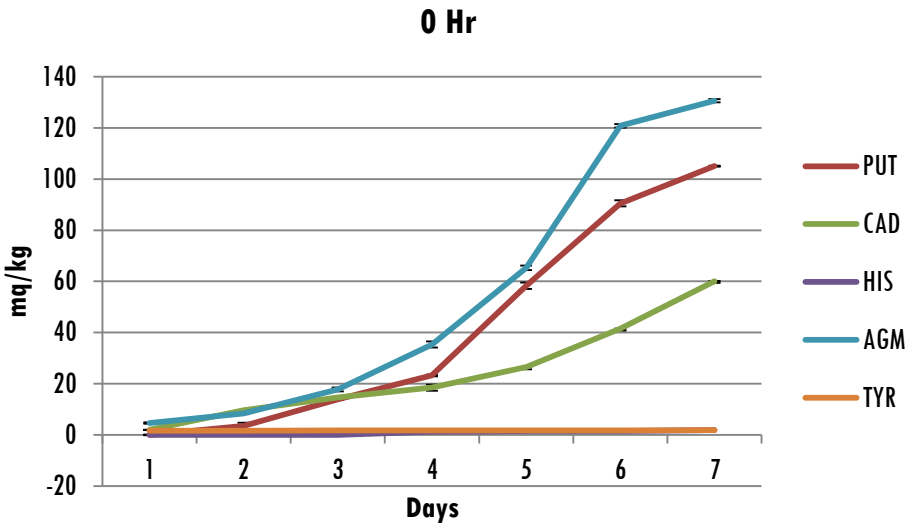
Histamine was not detected in any of the squid samples analyzed on the initial day. Histamine was observed only after 6th day in immediately iced lots

and the value is never exceeded 3 mg/kg in all lots. Staruszkiewicz et al., (2004) found no significant increases in histamine levels in samples of fresh fish held for short incubation periods. Moreover, histamine, the causative agent for scombroid poisoning, is not present in the flesh of fish when it is caught, but occurs when histamine-producing bacteria decarboxylate histidine to histamine during the spoilage process (Klausen& Huss, 1987; Morrow, 1991). However, the production of histamine is negligible in squid compared to the scombroid fishes, because of the low level of aminoacid histidine in squids (Yusuru 1992).

From the present study it is clear that PUT and CAD were the three most obviously changed amines in squid during ice storage. Increases in the amount of amines (PUT and CAD) in delayed iced squid sample exhibited more rapid than those at immediately iced sample. HIS and TYR levels increased gradually in low concentration during the storage period. However, spermine, and spermidine fluctuated within negligible levels during the storage period in all conditions. The appearance of high concentration of cadaverine, putrescine and agmatine between days six and 12 supports the view that bacterial activity contributes to spoilage at the late storage stage. The possible role of putrescine, cadaverine and agmatine in intoxication was not known according to Halasz et al. (1994), but they may act as a histamine poisoning potentiator. However, the low levels of histamine detected during the present experiment make such intoxication problems in squid irrelevant. The PUT, CAD, HIS, TYR can be use an excellent quality marker in squid, which agrees with Seyed Vali Hosseini, (2013).

Further studies will be needed to control or reduce the concentrations of biogenic amines in squid during storage, since these contents tended to increase with the increased storage days. The study also demonstrated a good correlation between the production of BAs and TMA and TVBN values as well as sensory rating.

a)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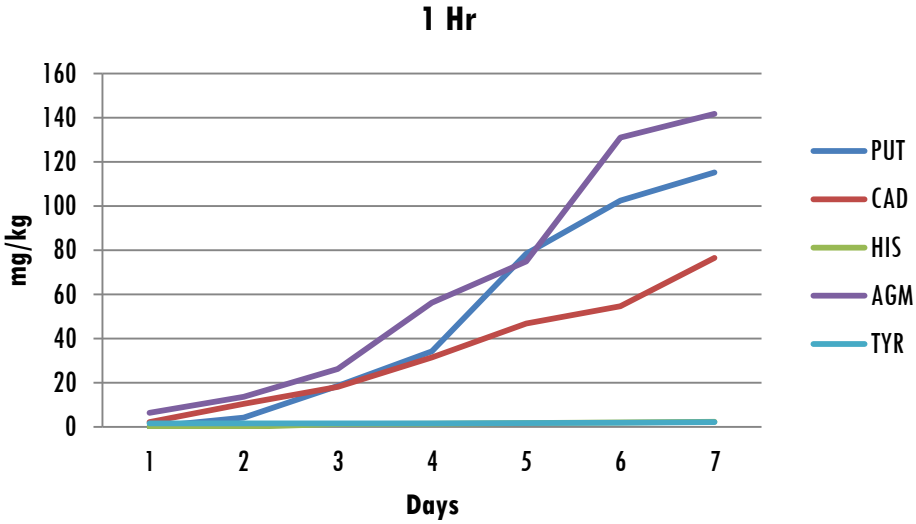
b)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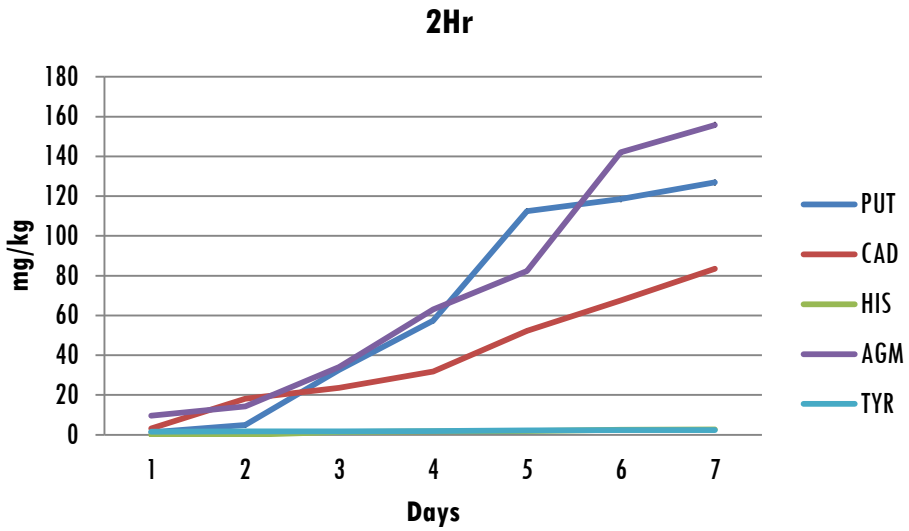
AGM
TYR

c)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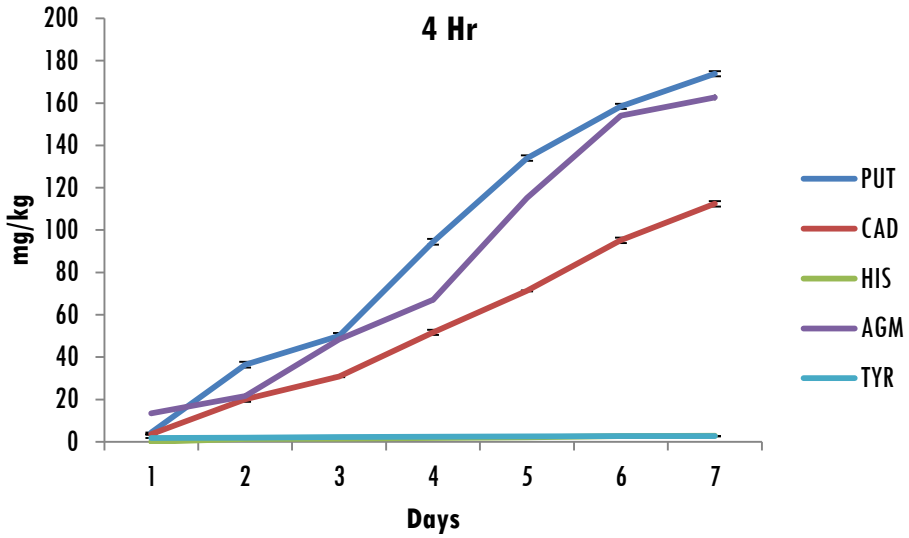
d)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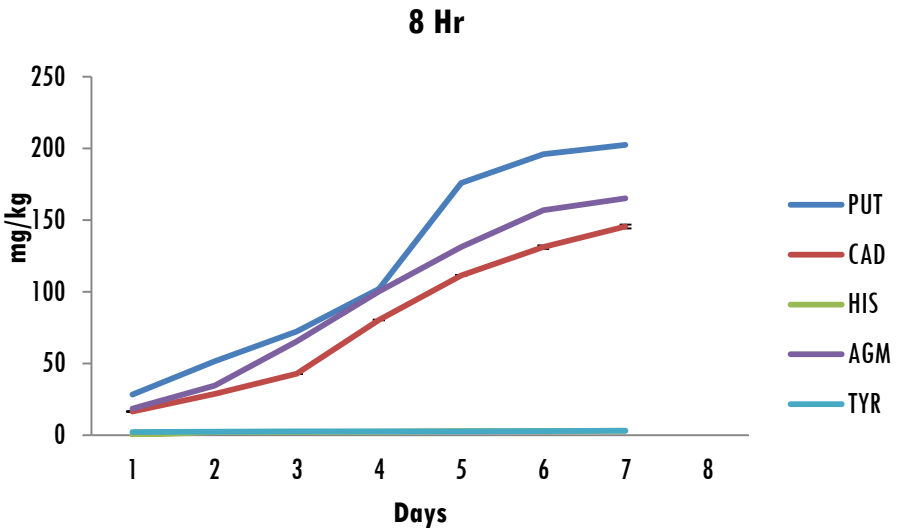

Fig: 6.2(a-e) Effect of delayed icing on the production of biogenic amines

6.4.2.2 Trimethyl amine (TMA)

TMA is the most common quality index employed in the fish industry for evaluating freshness and spoilage in marine fish, since it is produced from bacterial degradation of trimethylamine oxide (TMAO), a naturally occurring osmoregulatory substance found in most marine fish species (Koutsoumanis, 2002; Huss, 1995). The TMA content in fresh fish is reported to be 0.2-2mg/100g TMA-N (Govindan 1985). In the present study in squid the TMA was detected 1.3 and 1.9mg/100g in the initial day in immediately iced squid and iced after 1 hour respectively and it was significantly increased to 12.5 and 15.2 mg/100g during the storage period of 12 days which was just above than the recommended rejection value of 10mg/100g. The TMA of samples stored in ice 2, 4, and 8 hours after catch were 2.3, 2.8 and 8.6 mg/100g respectively at the 0^{th} day of storage. The changes in the TMA in squid ice stored after delaying icing for various time intervals are given in Table: 6-

1.TMA formation in the samples kept at high ambient temperature for 4 and 8 hours prior to icing were more advanced than the samples stored in the ice immediately after catch. The TMA value exceeded the acceptance limit in 10, 8, 6, 4 and 2 days respectively for 0,1,2,4, and 8 hour delay storage in ice. The quantity of TMA produced is, therefore, used to evaluate the activity of spoilage bacteria in the fish and is considered as a marker of the degree of spoilage.The absence (or extremely low levels) of production of TMA in some fish species during storage has been reported (Koutsoumanis, et al., 2002), and was attributedto the fact that *S. putrefaciens* population never reached levels of 10^8-10^9 CFU/g, a population considered crucialto the formation of TMA (Dalgaard, et al., 1993). Paulo Vaz-Pires et al.,(2008) reported in squid that the significant differences in TMA contents were observed only after 10 days of storage. Leblanc and Gill (1984) was detected an exponential production of TMA in *I. illecebrosus* squid stored at 2.5^0C. *In L. plei* TMA contents higher than 1 mg/ 100 g were found only after 12 days of iced storage (Lapa-Guimaraes et al., 2005).

Table: 6.1 Effect of delayed icing on TMA changes in *L.duvauceli* during subsequent ice storage

	TMA (mg/100 g)				
Days	**0hr**	**1hr**	**2hr**	**4hr**	**8hr**
0	1.3±0.05a	1.9±0.06b	2.3±0.20c	2.8±0.05d	4.3±0.05e
2	1.8±0.09a	3.5±0.07b	4.1±0.05c	6.9±0.05d	9.8±0.17e
4	4.2±0.08a	5.8±0.08lb	7.8±0.17c	11.0±0.15d	11.5±0.24e
6	5.3±0.11a	7.3±0.16b	10.2±0.07c	13.7±0.05d	14.7±0.24e
8	7.1±0.12a	10.4±0.11b	12.2±0.13c	16.3±0.14d	18.5±0.09d
10	9.7±0.21a	13.5±0.07b	14.3±0.24c	18.8±0.06d	21.2±0.14e
12	12.4±0.06a	15.2±0.12b	17.4±0.07c	22.4±0.06d	24.4±0.13e

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

6.4.2.3 Total volatile base nitrogen (TVBN)

TVB-N generally used to determine the degree of freshness of sea food during storage (Malle,et al., 1989). The significant increases in TVB-N levels during the storage day may be due to handling practices, resulting in bacterial growth. Protein and non protein nitrogenous compounds are formed as a result of microbial activity and tend to increase high microbial populations (Chitiri, et al., 2004). TVBN measures the total content of TMA, DMA, ammonia and other basic nitrogenous compounds. TMA is produced by spoilage bacteria, DMA by autolytic enzymes and ammonia by deamination of aminoacids, nucleotides and other volatile base compounds. The TVBN content of fresh squid was 7mg/100g, but squid iced after 8 hours increased to 16.4mg/100g. The changes in the TVBN of squid ice stored after delaying icing for various time intervals are given in Table 2. The result has indicated that the formation of TVBN value (59.5mg/100g on day 12 for 8 h delayed storage) is much rapid in samples exposed to high temperature for a longer period after catch. The low value of TVBN (7.1mg/100g) in the beginning of storage is an indication of high quality squid, where as the high value in delayed iced samples indicate the activity of autolytic enzymes and microbial spoilage. Considering 35 mg kg^{-1}as the acceptable level for TVBN, the lives for storage reached in 10-12, 8-10, 8, 4-6, 2-4 days respectively for 0, 1, 2, 4, hours delay of ice storage. There is a correlation between TVBN values and sensory score, where the TVBN values in samples exceeded the recommended values were in organoleptically unacceptable conditions. However, the present study suggests that the temperature control is very important to maintain the quality of squid.

Table: 6.2 Effect of delayed icing on TVBN changes in *L.duvauceli* during subsequent ice storage

	TVBN(mg/100 g)				
Days	**0hr**	**1hr**	**2hr**	**4hr**	**8hr**
0	7.1±0.11a	7.4±0.13b	10.3±0.05c	14.6±0.13d	16.5±0.05e
2	10.7±0.13a	14.5±0.13b	19.6±0.12c	21.4±0.05d	29.5±0.13e
4	16.2±0.32a	20.9±0.08b	24.7±0.08c	30.4±0.06d	35.5±0.07e
6	21.3±0.17a	27.4±0.13b	30.1±0.12c	38.7±0.32d	41.2±0.08e
8	26.3±0.06a	33.1±0.09b	35.4±0.19c	46.6±0.44d	49.7±0.18e
10	31.5±0.52a	38.5±0.05b	41.5±0.08c	51.3±0.14d	55.8±0.08e
12	36.3±0.08a	40.5±0.18b	44.4±0.33c	55.5±0.26d	59.5±0.13e

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly ($P<0.05$)

6.4.2.4 pH

Table 6-3 shows the effect of delayed icing on the changes in pH of the squid muscle during subsequent storage. The initial pH of the muscle was 6.54 which was increased to 7.21 after 12 days of storage in samples stored in ice immediately while the pH increased to 7.37, 7.42, 7.68 and 7.74 during storage in samples kept at ambient temperature for 1,2,4 and 8 hours prior to icing. There was marginal increase in pH due to delayed icing indicating the significance of temperature in freshness of squid. Based on sensory evaluation, thepH value above 7 was found unacceptable for consumption or spoiled. The increase in pH during ice storage was associated with the stage of rapid spoilage of the squid and reflected the production of alkaline products of autolysis and bacterial metabolites in the spoiling flesh (Vladimiros, et al., 2008). Accordingly, measurements of pH could provide an indicator of squid decomposition and spoilage.

Table: 6.3 Effect of delayed icing on pH changes in *L.duvauceli* during subsequent ice storage

	pH				
Days	**0hr**	**1hr**	**2hr**	**4hr**	**8hr**
0	6.54±0.01	6.57±	6.61±0.05	6.66±0.03	6.78±0.02
2	6.54±0.00	6.65±0.02	6.76±0.01	6.78±0.02	6.82±0.01
4	6.69±0.05	6.84±0.03	7.11±0.02	6.92±0.04	7.02±0.00
6	6.71±0.04	6.93±0.00	7.02±0.00	7.22±0.00	7.31±0.00
8	6.90±0.02	7.11±0.03	7.12±0.02	7.33±0.05	7.45±0.05
10	7.08±0.01	7.16±0.01	7.31±0.04	7.54±0.01	7.50±0.01
12	7.21±0.00	7.28±0.05	7.42±0.00	7.68±0.02	7.75±0.01

Values are expressed as mean±SD from three replicates with different superscripts in a column differs significantly (P<0.05)

6.4.3 Bacteriology

6.4.3.1 Total Plate Count (TPC)

The bacterial spoilage of aquatic lives indicated the moment the fish/shellfish is takes out the water. During ice storage, there was a continuous increase in total plate count in the squid till the end of the storage ($p < 0.05$) (Fig. 6-3). In fresh sample the total plate count was 3.36 log cfu/g which was increased to 6.72log cfu/g after 12 days of storage in immediately iced sample, while the total plate count increased to 7.1, 8.2, 9.04, 9.79 log cfu/g during storage in samples kept at ambient temperature for 1,2,4 and 8 hours prior to icing. Paarup et al., (2002b) reported that the plate count of squid mantles stored at 4°C increased from an initial level of 4 to 7 log cfu/g, when it was sensorially rejected. Pires and Barbosa (2004) found a bacterial load of 5–6 log cfu/cm^2 in octopus (*Octopus vulgaris*) stored in crushed ice at the point of rejection. Paarup et al., (2002) found that dominating microflora in squid stored at 4°C included motile, gram-negative rods. It is suggested that the spoilage of iced squid is likely to result from a combination of autolytic and bacterial changes (Civera, 2000). TPC increased with increasing delayed ice

storage time and decrease the quality of squid, which agrees with Prafulla et al., (2000). If 6 log CFU/g is taken as the TPC limit of acceptability, therefore; the shelf-life of squid (*Loligo duvauceli*) was approximately 10, 8, 6, 4 and 2 days at ambient temperature for 1, 2, 4, 6 and 8 hours prior to icing.

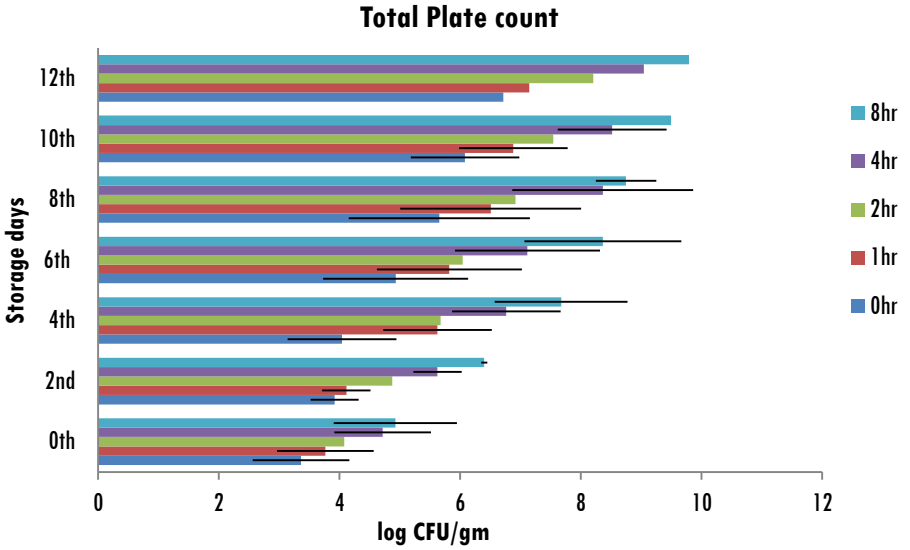

Fig. 6.3 Effect of delayed icing on TPC (log cfu/gm) during storage

6.4.3.2 Psychrophilic count

Psychrophiles loads of whole squid and changes that occurred during storage in ice are presented in Fig.6.4. The count was 2.5 log cfu/g and which was increased to 6.3log cfu/g after 12 days of storage in immediately iced sample, while the psychrophilic count increased to 6.6, 7.1, 8.5, 8.9 log cfu/g during storage in samples kept at ambient temperature for 1, 2, 4 and 8 hours prior to icing. In sample iced after 8 hour, the initial count was 4 log cfu/g, which was drastically increased to 8 log cfu/g at the end of the storage day. Lapa-Guimaraes et al. (2002) observed a psychotropic bacterial count of 6 log cfu/g in squid (*Loligo plei*) after 16 days of storage in ice.

It is reported that most of the microorganisms have the capacity to produce biogenic amine, including Gram positive and Gram negative bacteria of different genera and species, such as *Bacillus, Salmonella, Citrobacter, Clostridium, Escherichia Klebsiella, Proteus, Shigella,, Photobacterium, Pseudomonas* and the *lactic bacteria Lactobacillus, Streptococcus and Pediococcus* are able to decarboxylate one or more amino acid (Brink et al., 1990; Kim et al., 2001), although such capacity is generally depend on a strain-level characteristics of such organism (Rodwell et al., 1953). Biogenic amine production by bacteria was based on the availability amino acids in food and decarboxylases synthesizing capacity of organism (Bover, 2000).

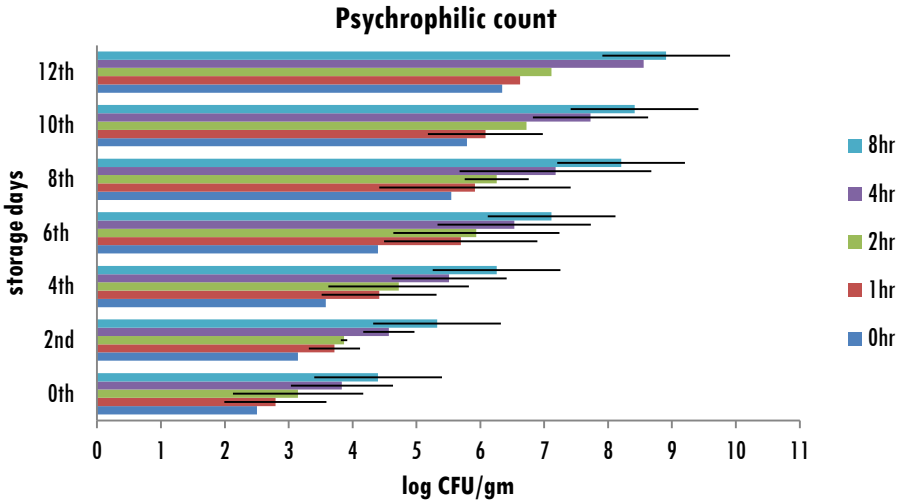

Fig. 6.4 Effect of delayed icing on psychrophilic count (log cfu/gm) during storage

6.5 Conclusions

The BAs in common squid stored in ice under five different conditions were evaluated and the data showed that there is no important risk of HIS and TYR as the more dangerous amines in food because their levels were under the limit and negligible over the period. However, all BAs increased during the storage, the values of PUT and CAD was clearer than the others and it can

indicate that these amines can be good markers to evaluate squid quality. It is indicated that the temperature and the duration of storage after catch significantly affected on the levels of BAs and generally the levels in samples with 8 h delay before icing was significantly higher. TMA, TVBN Sensory evaluation and microbial counts, as in other works with fish and with squid, can still be considered useful additional data to follow the degradation process.

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Chapter

7

DEVELOPMENT OF QUALITY INDEX IN INDIAN SQUID (*Loligo duvauceli*) BASED ON BIOGENIC AMINE FORMATION

Contents

7.1 Introduction

Biogenic amines (BA), the non-volatile, low molecular weight nitrogenous compounds are formed through the microbial decarboxylation of free amino acids in aquatic products poses health concern to consumers (Marine, et al., 1995; Lehane,et al.,2000; Chong, et al., 2011). Low levels of BA in food products do not present anadverse effect to human health because of the presence mono or diamine oxidases enzymes in human intestine can rapidly detoxify the amines. However, the ingestion of aquatic foods containing high levels of BA may result in severe toxicological symptoms (Mohamed, 2010), partially in individuals where enzyme is genetically deficient (Wantke, et al., 1994; Wantke, et al., 1996) or due to intake of alcohol, or due to monoamine oxidase inhibiting drugs (Wantke, et al., 2001).Thus, undesirable effects, including intestinal symptoms, nausea and

headaches occur after the consumption of food that contains BAs, especially histamine, even at low levels.The consumption of fish containing 200 mg/kg of histamine (HIM) or so was reported to cause the toxicological symptoms of ‘‘scombroid food poisoning’’ (FSSAI). Toxicity of HIM may be potentiated by the presence of putrescine (PUT) and cadaverine (CAD) (Smith, 1981; Stratton, 1991). Consuming high levels of tyramine (TYM) in food called ‘‘cheese reaction’’, leads to uncontrolled hypertension symptoms (Bardocz, 1995; Benedetti, 1983). Because of the toxicological effects of BA on human health, regulations and legal requirements for the maximum limits of BA (especially for HIM) in aquatic products have been suggested and established are explained in previous chapter (Chapter 2, review of literature Table.2.).

The formation of BA in aquatic products depends on many factors, such as the contents of free amino acids, the presence of bacterial amine decarboxylases, and the storage conditions of the aquatic products. In fact, high levels of BA in aquatic products are usually found to be due to inadequate preservation, in which BA can be largely formed through the microbial decarboxylation of amino acids by various specific amine-forming enzymes (Park, et al., 2010). Therefore, the contents of BA in aquatic products may strongly be influenced by the freshness of raw materials and the environmental storage conditions, especially storage temperature and time (Carelli, 2007).There is a definite time-temperature leading to the formation of BA in seafoods.

Some of the quality indices used to assess freshness or degradation in fish have shown to be inadequate when applied to squid. Freshness indicators such as K value, total volatile basic nitrogen (TVBN), and pH (Ohashi et al., 1991) are found not suitable in cephalopod species, as some of the metabolic pathways are different from that of fish. Few authors have recommended

agmatine (Yamanaka et al., 1987; Ohashi et al., 1991), microbial counts of psychrophilic bacteria like *Photobacterium phosphoreum* and *Pseudoalteromonas* (Paarup et al., 2002) as quality indicesor spoilage indicators in cephalopods. Civera et al., (1999) reported even the counts of specific spoilage indicators (H_2S producers) to be appropriate to determine the early stage of deterioration in cephalopods.

The prospect of the export of Indian squid to overseas has resulted in their increased exploitation. Although the export of squid is gaining momentum in India, the processing industry faces severe problems due to the rapid spoilage and development of off odour in chilled and frozen squid, which has led to frequent rejection of commodities by the importing countries like USA (FDA, 2013). Examination of routine microbiological as well as biochemical quality indices prescribed for fish by the regulatory authorities has failed to judge the decomposition status of squid. This industrial problem was hence taken up with the objectives to derive a suitable biochemical index for the iced or chilled Indian squid, *Loligoduvauceli*, when subjected to different storage conditions in relation to its sensory and microbial changes, so as to evolve effective strategies for controlling their spoilage. The objectives of the present study were to investigate the changes in the concentration of BA with respect to the squid quality.

It has been established in the previous chapters (Chapter 4, 5, 6) that the production of biogenic amines especially PUT, CAD, HIM, TYM and AGM increased with increase in storage period at different storage conditions. Therefore, the present study aims to find out the relationship between total biogenic amines and quality of squid and to correlate the sensorial scale with quantitative indicators of freshness, safety and spoilage

7.2 Review of Literature

7.2.1 Quality index in cephalopods

Cephalopods have a spoilage pattern different from other species, being dominated by autolysis, leading to a shorter shelf-life based on sensory characteristics (8–10 days), and a very late microbial growth, approximately 10^6cfu/g on day 16 in ice (Hurtado et al., 2001; Lapa-Guimaraes et al., 2002). They undergo very rapid spoilage due to the action of endogenous and bacterial proteases. Autolytic activity of cephalopod muscle is around six times greater than fish muscle (Matsumoto, 1980; Jimenez and Borderias, 1983). Protease plays an important role in the breakdown of protein and its activity in cephalopods is higher than in various species of fish (Hurtado et al., 1999). These muscle proteases belong to the sarcoplasmic proteins of the myosystem.

Cephalopods spoil more rapidly compared with other fishes, mainly because they are smaller in size and their guts are not removed immediately after harvesting (chapter 4). So they are prone to early autolytic spoilage. Their biological and chemical composition of the tissue also supports rapid spoilage (Early and Stroud, 1982). The digestive gland of squid possesses strong proteolytic activity (Hatate et al., 2000) than that of various species of finfish, and it is possible that autolytic enzymes and NH_3 diffuse from gland to mantle during storage. It was reported that the maximum autolytic activity in cephalopods was 15-fold higher than in Pacific whiting, a fish well known to contain high levels of endogenous proteases (Hurtado et al., 1999). Squid (*Todaropsis eblanae*) was reported to contain 3.5-fold higher autolytic activity than blue whiting (Ayensa et al., 1999). Spoilage in cephalopods is dominated by autolysis that leads to a shorter shelf life by sensory characteristics (Lapa-

Guimaraes et al., 2002). The high proteolytic activity post mortem produces an increase of the levels of muscle-derived nitrogen components (Hurtado et al., 1999).

The NPN compounds so far identified from cephalopods include free aminoacids, betaines, TMAO, TMA, agmatine, adenine, adenus phosphoric acid, choline, hypoxanthine, methyl, and urea (Amano, 1951). Joseph et al. (1977) have recorded a decrease in NPN compounds from 682.3 to 133.6 mg/100g (80% loss) in squids held for 6 days in ice. Ragunath (1984) confirmed the loss of NPN and reported that the nitrogen from the body is leached out inthe ice melt water during ice storage of squid, *Loligo duvauceli*. Much lower losses of NPN recorded in iced squid are 40% in *Dosidicus gigas* (Romo et al., 1996), 69% in *Loligo plei* (Lapo-Guimaraes et al., 2005) and 79.5% in *Loligo duvanceli* (Prafulla et al., 2000). Breakdown of simple non-protein nitrogen compounds produces off-odors and flavors in crustaceans (Adams and Moss, 1995).

The α-amino nitrogen has been suggested as one of the major quality indices of the meat quality and extent of breakdown of peptide bonds (Nagaraj, 1994). The content of nitrogen from free amino acids (FAA-N) in squid, *Loligo peli* represented 36.8% of NPN and the NPN fraction represented 31.7% of the total nitrogen (TN) (Sikorski and Kolodziejska, 1986). Otsuka et al., (1992) also reported that the total content of FAA in squid, *Doryteuthis bleekeri* species stored in plastic bags in ice decreased after the first day, but afterwards remained stable until 15 days of storage. Romo et al. (1996) reported that squid, *Dosidicus gigas* had lost 40% of FAA after 72 h of storage in ice.

Ammonia has been identified as an excellent indicator of squid quality (LeBlanc and Gill, 1984). Increase in the ammoniacal nitrogen was also observed in iced squids on storage (LeBlanc and Gill, 1984; Paarup et al., 2002). The shorter lag phase and the faster rate of production of ammonia compared to TMA is probably related to autolytic activity, which is considered mainly responsible for squid spoilage (LeBlanc and Gill, 1984). Licciardello et al. (1985) observed a final exponential increase of NH_3 in whole iced squid, *Loligo peli*. The increase in ammonia was mainly attributed to the bacterial deamination of proteins, free amino acids and other basic nitrogenous compounds in the flesh of cephalopods (Huang et al., 1993; Lougovois et al., 2008). Strong ammoniacal off-odours have also been described as the main reason for sensory rejection of conventionally iced giant squid, *Todarosuse blanae* (Paarup et al., 2001).

Lipids undergo hydrolysis presumably due to the activity of lipases and phospholipases, resulting in the accumulation of FFA during iced storage of cephalopods (Lovern, 1962). Hydrolytic rancidity could therefore be a major problem in ice-stored octopus, as lipolytic enzymes could also be derived from psychotropic microorganisms (Huis in't veld, 1996). Increase in FFA has been reported during ice storage of cuttlefish (Sophia and Sherief, 2003). Subramaniyan (2006) reported an increase in FFA values from 0.80 to 3.2 % as oleic acid in cuttlefish during ice storage. Venkatappa and Dhananjaya (2006) reported an increase in FFA values from 4.57 to 31.72% as oleic acid in cephalopod held in ice for 19 days.

TVBN values are the suitable indicators for the initial decomposition of squid (Yamanaka et al., 1987). Increase in TVB-N contents was also observed by several authors in iced cuttlefish (Chidanada et al., 1986; Sastry, 1981; Sophia and Sherief, 2003) and octopus (Venkatappa and Dhananjaya, 2006;

Atrea et al., 2009). Uchiyama and Ehira (1974) attributed that the increases in TVB-N and TMA-N in asceptic muscle is due to deamination of nucleotides. Ke et al. (1984) had proposed a classification to grade the quality of cephalopods in accordance with TVB-N content, where the limit of <30mg for A grade and 30-45 mg for B grade and >45mg is considered unacceptable. Moral (1997) found that the iced squid, *Todarosuseblanece* became unacceptable, when TVB-N exceeded 45mg/100g. Contradictory views do exist in using TVB-N as a freshness indicator in certain cephalopods mainly because of the presence of a different metabolic pathway in cephalopods (Ohashi et al., 1991). The acceptable limits of TMA-N and TVB-N therefore depend on the species and storage condition (Paarup et al., 2002).

Bacteriology of iced squid, Loligo spp. was studied by Joseph et al. (1977). Civera (2000) has examined the chemical and microbial characteristics of cephalopods. Prafulla et al. (2000) have noticed an increase (4 to 6 log cfu/g) in TPC of squid and cuttlefish held under chilled condition by different methods of icing. Increase in the psychrophilic bacteria from 2 to 5 log cfu/g in the squid, *Loligo plei* stored either in contact or non-contact ice was reported by Lapa-Guimaraes (2002). Paarup et al. (2002) studied the sensory, chemical and bacteriological changes during ice storage of squid, *Todaropsis eblanae*, and they noticed an increase in TPC from 3 to 7 log cfu/g. Lapa-Guimaraes et al. (2005) observed an increase in the psychrophilic bacteria from 4 to 6 log cfu/g during storage of iced squid, *Loligo plei*. Sulphide-producers have been reported to be the main spoilage organisms of whole iced octopus (Vaz-Pires and Barbosa, 2004), whereas strains belonging to the genus Pseudo alteromonas appear to dominate the spoilage of whole squid during iced storage (Paarup et al., 2002).

7.2.2 Biogenic amines index

Biogenic amines are naturally present in very low levels in fresh fish and the presence of high amounts of these compounds is associated to bacterial degradation (Ozogul and Ozogul, 2006; Simat and Dalgaard, 2011; Cunha et al., 2013). Assessing biogenic amine presence is important not only from a toxicological point of view, but because these substances can be used as indicators of food degree of freshness or spoilage (Alberto et al., 2002; Ozogul and Ozogul, 2006; Onal, 2007; Park et al., 2010; Sagratini et al., 2012). Mietz and Karmas (1977), in the case of canned tuna that during chilling the putrescine, cadaverine and histamine content increased, while spermine and spermidine decreased compared to fresh squids. Therefore, they suggested a Chemical Quality Index (QI) based on the concentration of the following biogenic amines:

$$Quality\ Index\ (QI) = \frac{Histamine + Putrescine + Cadaverine}{Spermine + Spermidine}$$

The authors observed that the quality index increased when the sensory scoring of the canned product decreased. Thus, they suggested that a product with QI below 1 would have been processed with a first quality raw material, while those with values above 10, would indicate a raw material with very poor microbiological quality.

Veciana-Noguez et al. (1997) assessing tuna stored at 0, 8 and 20ºC for 21, 9 and 4 days respectively, concluded that spermidine and spermine contents are not indicators of quality loss and that significant alterations in histamine, putrescine, cadaverine and tyramine levels during the storage temperatures were noticed during the storage period. In addition, they observed that sensory rejection of samples kept at 8ºC occurred on the 5th storage day, although the QI value of 10 proposed by Mietz and Karmas (1977) had not been attained. These results

support the proposal of a Biogenic Amine Index (BAI) based on the sum of histamine, cadaverine, putrescine and tyramine levels considering values below 50 mg kg^{-1} as indicative of good quality food.

The biogenic amines indices from different fish species is listed in Table 1. Krizek et al. (2002) found that the Quality index proposed by Mietz and Karmas (1977) has little application to assess the quality of carp (*Cyprinus carpio*) stored in non-hermetic packages kept at 3 and 15ºC for 13 and 4 days respectively, due to insignificant decrease in spermine concentration. Putrescine content presented the best correlation with the sensory quality of the meat. Based on these results, the authors observed that good quality samples presented putrescine content up to 10 mg kg^{-1}, acceptable quality between 10-20 mg $kg^{-1}$and undesirable quality above 20 mg kg^{-1}. Histamine and cadaverine formation kinetics were similar to putrescine formation kinetics, however histamine content increase was observed only in evidently spoiled samples. It is, therefore considered, that the sum of putrescine and cadaverine contents might be used to assess carp quality. Ozogul and Ozogul (2006) assessed biogenic amine concentration in sardines (S*ardina pilchardus*) kept at 4ºC in the air, packed under modified atmosphere (60% CO_2 and 40% N_2) and under vacuum. From QI calculation proposed by Mietz and Karmas (1977) and BAI suggested by Veciana-Nógues et al. (1997), they observed that both indices increased as a function of storage time and showed good correlation with the sensory alterations of the samples. The QI 10 established as the acceptance limit was attained in 4, 8 and 12 of storage days in samples kept in the air, vacuum packed and packed under controlled atmosphere, respectively, times when the samples were sensory rejected. Bakar et al. (2010) studied biogenic amine content in barramundi (*Latescalcarifer*) stored at 0ºC and 4ºC for 15 days and calculated QI and BAI according to the formulas proposed by Mietz and Karmas (1977) and Veciana-Nógues et al.

(1997). The results showed that both indices increased during storage time and therefore can be used to determine the degree of spoilage of this species. Other freshness indices have been pointed out for different fish species based on the correlation of biogenic amine increased with storage period, such as cadaverine concentration in salmon (*Salmo gairdneri*) (Yamanaka et al., 1989), cadaverine and agmatine content in smooth weakfish (Ruiz-Capillas and Moral, 2001) and putrescine and cadaverine content in trout (*Oncorhynchus keta*) (Rodrigues et al., 2013).

Table 7.1 Biogenic amines indices in samples fish

Sample fish	Scientific name	Biogenic amines indices	References
Tuna		HI + PU + CA / SM + SD	Mietz and Karmas (1977)
Tuna	*Thunnusthynnus*	HI + CA + PU + TY	Veciana-Noguez et al. (1997)
Carp	*Cyprinus carpio*	PU and CA	Krizek et al. (2002)
Salmon	*Salmo gairdneri*	CA	Yamanaka et al. (1989)
Sardine	*Sardinapilchardus*	HI + PU + CA / SM + SD and HI + CA + PU + TY	Ozogul and Ozogul(2006)
Barramundi	*Latescalcarifer*	HI + PU + CA / SM + SD and HI + CA + PU + TY	Bakar et al. (2010)
Hake	*Merluccius merluccius*, L	CA and AG	Ruiz-Capillas and Moral (2001)
Trout	*Oncorhynchus keta*	PU and CA	Rezaei et al. (2007) and Rodrigues et al. (2013)

7.3 Materials and Methods

7.3.1 Quality index and biogenic amines index

The quality index (QI)and the biogenic amine index (BAI) were calculated according to the procedures described by Mietz and Karmas (1977) and Veciana-Nogues et al. (1997) respectively. The formulae used were as follows:

$$Quality\ Index\ (QI) = \frac{Histamine + Putrescine + Cadaverine}{Spermine + Spermidine}$$

$$Biogenic\ Amine\ Index\ (BAI) = Histamine + Putrescine + Cadaverine + Tyramine$$

The data (Biogenic amines and Total plate count) from previous chapters (Chapter 4, 5 and 6) were used to find out the quality index and the biogenic amine index

7.3.2 Statistical analysis:

Using IBM SPSS Statistics software, a polynomial curve was found to fit the experimental data of the TPC and the amine concentrations during storage; its formula was as follows:

$$Y = -A_1x^4 + A_2x^3 - A_3x^2 + A_4\,x - x$$

Where: y is the concentrations of amines (mg kg^{-1}), x is the TPC (Total Plate Count) values (log CFU g^{-1}). A_1 and A_2 are the parameters which can be obtained by the fitted polynomial curve. The regression coefficient (R^2) values of the fitted polynomial curves were used to indicate the correlations between the bacterial growth and the amine formation during storage.

7.4 Results

In general, the use of more than a single BA i.e., a BA index that consists of a combination of BAs is recommended to avoid the limitation of a possible variability in the content of one amine and seems more appropriate as a quality indicator. The index of Mietz and Karmas (1981) considers the increases in putrescine, cadaverine, and histamine levels with decreases in spermidine and spermine, and the index described by Veciana-Nogues et al. (Veciana-Nogues, et al., 1997) for tuna, which includes putrescine, cadaverine, histamine, and tyramine (Table 7.1).

In the present study (Result from chapter 4) the contents of BAs at the point of rejection by sensory and microbial limiting values of gutted squid stored in ice (12 to 14 days) were within the range of 50.9 to 70.3 mg/Kg for cadaverine, 102 to 115 mg/Kg for putrescine. 1.3 to 1.6 for histamine, and 2 to 2.1 mg/Kg for tyramine. But in the case of whole squid contents of BAs at the point of rejection by sensory and microbial limiting values were within the range of 48 to 64 mg/Kg for cadaverine, 98.5 to 123 mg/Kg for putrescine and 1.3 to 1.6 mg/Kgfor histamine, and 2.2 to 2.4 mg/Kg for tyramine. The contents of histamine at the time of rejection in squid were lower than those reported in tuna (Veciana-Nogues, et al., 1997). However, the levels of the other BAs, such as putrescine, cadaverine, and tyramine, in squid were slightly higher than those described for tuna (Veciana-Nogues, et al., 1997). Agmatine concentration showed an increasing trend from 0 day onwards and reached a maximum on the 12^{th} day (141.6 mg/Kg) and thereafter a decline was observed. A similar trend was also observed in whole squid during storage. This indicates that squid is also shows enough BA accumulation especially PUT, CAD, HIS, and TYR during storage to be considered suitable as a freshness or quality indicator.The quality index and the biogenic amine indexes proposed by Mietz and Karmas (1981) and Veciana-Nogues et al. (1997) were calculated and presented in table 7.3. Table (7.2&7.3) shows the values obtained from the index of Veciana-Nogues et al. (1997) (putrescine plus cadaverine plus histamine plus tyramine) were shows more satisfactory value and strong correlation with BA concentration and microbial flora, whereas the score obtained for the index (Mietz and Karmas, 1981) were shows weak correlation (Fig.7-2). This index contemplates the four amines (putrescine plus cadaverine plus histamine plus tyramine) that are most relevant in squid spoilage from the quantitative and qualitative point of view. Table 7-3 shows the evolution of the BA index observed in different storage

temperature study performed, which followed a gradual increase during ice storage. According to the BAI described by Veciana-Nogues et al. (Veciana-Nogues, et al., 1997) a BA index limit of acceptability may be established in a range of 150-230mg/Kg based on the total plate count and sensory data.

Because of the significant increases ($p < 0.05$) of BA especially for PUT, CAD, HIM and TYM in squid, the changes of microbial flora in the squid were analyzed to investigate the correlation between the growth of bacteria and the formation of BA during ice storage at different storage conditions (Table 7-4). The results in (chapter 4, 5 & 6) showed that the counts of TPC were found to significantly increase ($p < 0.05$) with increases of storage time in Squid at different storage conditions. A polynomial curve was employed to fit the relationship between the TPC and the amine formation based on the experimental data, in which the regression coefficient (R^2) values were used to reflect the correlations between the TPC and the concentrations of BA especially PUT, CAD, TYR, and HIS during storage. As shown in Table 7.4, strong positive correlations ($R^2 > 0.9$) existed between the TPC and the concentrations of PUT, CAD, HIM and TYM in squid during storage at different treatments. Similar results ($R^2 > 0.9$) were also observed in (Figure: 7-1(a-f) individual biogenic amine (PUT, CAD, AGM, HIS, TYR) except SPERM and SPERMD in gutted squid at 4^0C. PUT and CAD are the most common BA in aquatic products, but the two amines do not directly present serious toxicological effects on human health. However, it has been proved that the occurrence of PUT and CAD in aquatic products can potentially promote the toxicological effects of HIM and TYM, through inhibiting histamine metabolizing enzymes, such as monoamine or diamine oxidase and histamine methyl transferase(Smith, 1981; Stratton et al., 1991). In addition, the occurrence of PUT and CAD in foods can react with nitrite to form heterocyclic carcinogenic nitrosamines, which are some of the most important human carcinogens (Park et al., 2010; Santos, 1986).

Table: 7.2 Changes in Biogenic amines (BA)(mg/Kg), QI, BAI TPC (log CFU/g) and sensory demerit score in gutted and whole *L.duvauceli* for storage at 4^0C. The results are the mean of three replications± the standard deviation

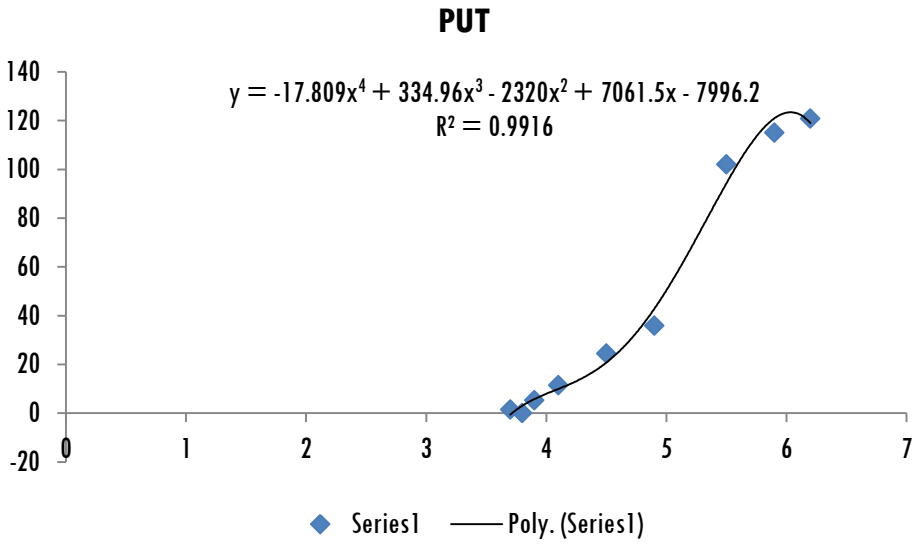
Changes in Biogenic amines (BA)(mg/Kg), QI BAI TPC (log CFU/g) and sensory demerit score in gutted sample											
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPERM**	**QI**	**BAI**	**TPC**	**Sensory**
0	ND	2.5±0.76	ND	4.7±1.20	1.1±0.30	5.2±1.01	265±2.01	282.7±1.9	3.6±0.8	3.8±0.06	0
2	1.4±0.17	4±0.32	ND	12.4±0.37	1.2±0.12	5.6±1.11	252±2.01	254.1±1.8	6.6±0.4	3.7±0.56	2.1±0.99
4	5.2±1.04	6.5±1.20	ND	19.8±1.20	1.3±0.03	3.4±1.12	220±2	227.1±0.9	13.0±2.2	3.9±0.23	5.4±0.74
6	11.4±1.22	11±1.05	ND	24.3±1.20	1.3±1.02	3.2±1.01	227±1.98	241.8±4.2	23.7±1.1	4.1±0.12	7.4±0.52
8	24.4±0.51	24.4±1.20	ND	40±1.14	1.4±0.03	3.3±1.01	251±1.9	282.8±3.8	50.2±1.7	4.5±0.05	9.4±0.52
10	35.8±1.50	37.3±1.21	1.3±0.14	77.2±1.36	1.7±0.01	3.5±1.14	240±2.1	287.7±3.5	76.0±2.8	4.9±0.13	13.9±0.64
12	102±1.53	55.9±1.20	1.3±0.20	141.6±1.20	2.0±0.07	2.5±1.01	230±2.2	355.6±2.7	161.1±2.6	5.5±0.04	15.8±0.46
14	115±1.51	70.3±1.56	1.6±0.03	124.4±1.78	2.1±0.03	3.9±0.59	245±1.08	379.6±2.0	189.0±3.0	5.9±0.06	16.0±0.00
16	120.8±0.67	80.9±1.29	1.7±0.01	125.2±1.28	2.3±0.03	3.2±0.59	244±2.31	390.9±2.5	205.6±1.1	6.2±0.91	16.0±0.00
Changes in Biogenic amines (BA)(mg/Kg), QI, BAI in whole squid sample											
Days	**PUT**	**CAD**	**HIS**	**AGM**	**TYR**	**SPERMD**	**SPERM**	**QI**	**BAI**	**TPC**	**Sensory**
0	ND	2.5±0.76	ND	4.7±1.20	1.1±0.30	5.2±1.01	265±2.1	265.4±2.0	3.6±0.8	3.8±0.41	00
2	3.6±0.26	2.7±0.33	ND	23.5±1.88	1.2±0.02	5.1±0.11	257±2	261.1±2.0	7.4±0.5	3.8±0.07	3.8±0.46
4	12.9±0.62	11.9±1.27	ND	65.2±1.36	1.4±0.76	2.5±0.30	254±1.8	271.6±2.7	26.2±1.9	4.5±0.06	9.9±0.64
6	21.2±1.34	16.7±1.26	ND	73.4±1.36	1.3±0.24	2.2±0.32	252±0.95	280.7±1.7	39.2±0.5	4.7±0.15	12.4±0.92
8	37.6±1.32	31.7±1.27	1.1±0.07	159.8±0.31	1.6±0.76	3.0±0.30	243±2.7	292.2±0	72.0±2.7	4.9±0.04	14.6±0.74
10	98.5±1.21	48.4±1.87	1.3±0.20	135.7±2.70	2.2±0.20	4.6±0.35	236±±2.5	346.3±2.5	150.6±2.9	5.6±0.06	15.9±0.35
12	123.3±1.34	64.3±1.2	1.6±0.14	133.5±1.23	2.4±0.04	6.8±0.24	224±0.45	358.3±2.4	231.4±3.5	5.9±0.34	16.0±0.00
14	195.6±1.28	90.2±1.25	1.7±0.20	129.8±1.36	2.5±0.02	6.3±0.35	232±0.59	443.6±2.4	290.0±2.9	6.3±0.21	16.0±0.00
16	210.0±1.87	98.4±1.27	1.9±0.11	129.5±0.82	2.8±0.02	6.7±0.02	241±0.23	467.5±2.3	313.1±2.8	6.4±0.74	16.0±0.00

Table 7.3: Changes in Quality Index (QI) and Biogenic Amine Index (BAI) in *L.duvauceli*, stored at different temperature. The results are the mean of three replications

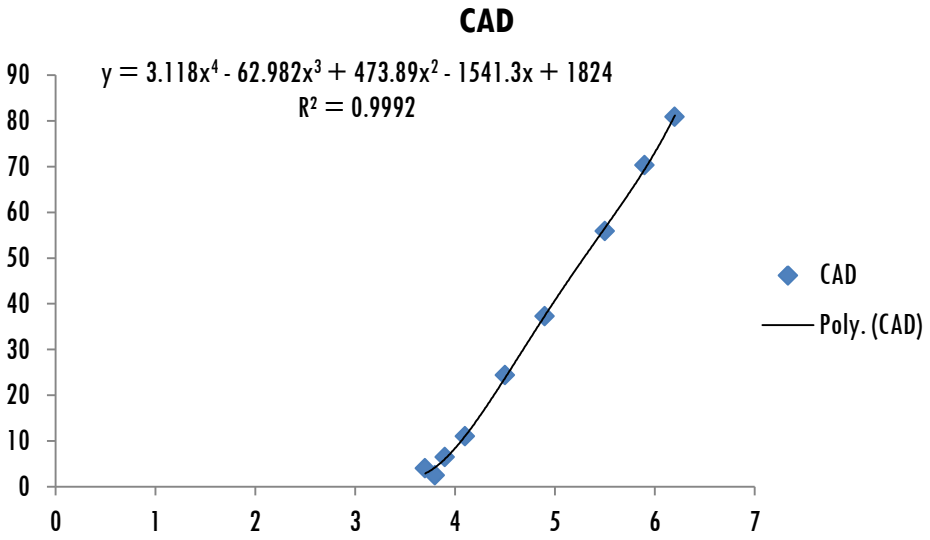
0±2°C			4±2°C			30±2°C			-18±2°C		
Days	QI	BAI	Days	QI	BAI	Hour	QI	BAI	Months	QI	BAI
0	233.1±1.1	1.4±1.2	0	233.3±0.7	1.3±0.4	0	233.1±0.6	1.5±1.4	0	233.1±1.3	1.3±0.4
2	237.2±1.3	6.9±0.8	2	260.6±0.8	15.2±1.4	6	169.5±0.4	43.1±2.2	2	235.0±1.2	1.4±0.6
4	246.0±0.9	19.3±1.4	4	249.1±0.5	51.5±0.5	12	300.5±1.2	163.1±1.5	4	236.7±1.6	4.5±1.2
6	258.5±2.1	40.5±1.2	6	290.5±1.2	125.3±1.1	18	292.3±1.3	215.1±2.2	6	241.3±1.2	9.3±1.0
8	265.8±2.0	95.3±2.1	8	278.1±1.7	174.7±1.8	24	223.4±0.5	250.2±0.5	8	243.4±1.3	18.7±0.5
10	274.8±1.8	127.4±2.2	10	316.5±1.5	223.8±1.5	-	-	-	10	247.9±0.6	25.0±0.3
12	298.1±1.4	177.5±1.5	12	315.7±0.8	263.4±1.1	-		-	12	254.3±1.2	37.5±1.2
14	311.3±0.7	242.6±1.3	14	362.0±0.6	290.1±1.2	-	-	-	-	270.8±0.9	50.5±0.5

Table 7.4 Correlations between the microbial flora (TPC) and the biogenic amine index in *Lologo duvauceli* during different storage temperature

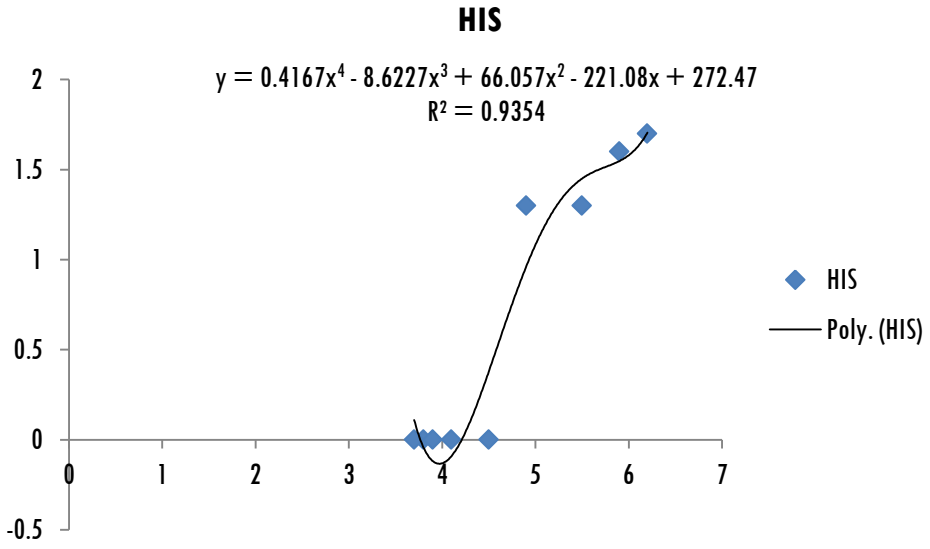
Storage condition	Microbial flora	Total Biogenic amine(R^2)
Gutted/ Whole (4±2°C)		
Gutted	TPC	0.998
Whole	TPC	0.995
Different storage temperature		
30±2°C	TPC	0.978
4±2°C	TPC	0.981
0±2°C	TPC	0.988
-18±2^{0}C	TPC	0.971
Delayed icing		
0 hr	TPC	0.994
1 hr	TPC	0.994
2 hr	TPC	0.995
4 hr	TPC	0.976
8 hr	TPC	0.985



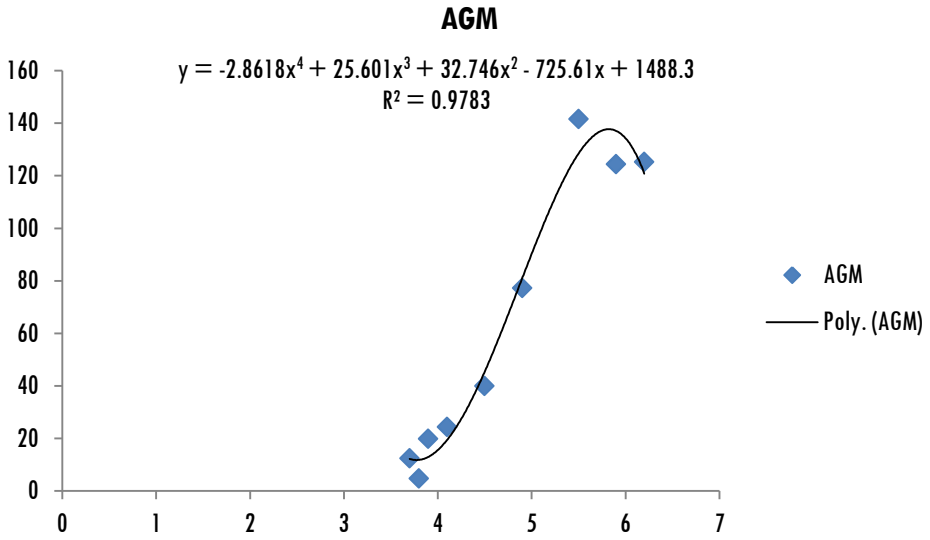
a)


CAD
y = 3.118x⁴ - 62.982x³ + 473.89x² - 1541.3x + 1824
R² = 0.9992
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
CAD
Poly. (CAD)

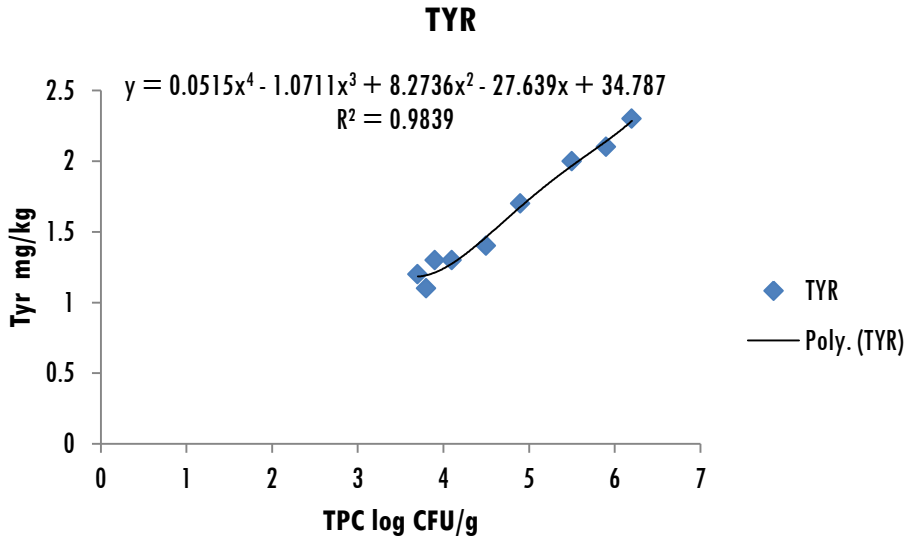
b)


HIS
y = 0.4167x⁴ - 8.6227x³ + 66.057x² - 221.08x + 272.47
R² = 0.9354
2
1.5
1
0.5
0
-0.5
0
1
2
3
4
5
6
7
HIS
Poly. (HIS)

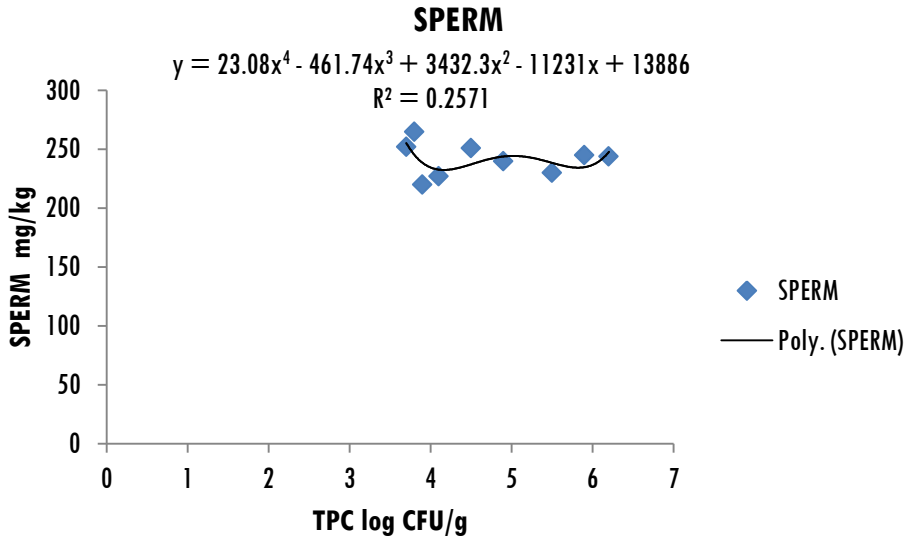
c)


AGM
y = -2.8618x⁴ + 25.601x³ + 32.746x² - 725.61x + 1488.3
R² = 0.9783
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
AGM
Poly. (AGM)

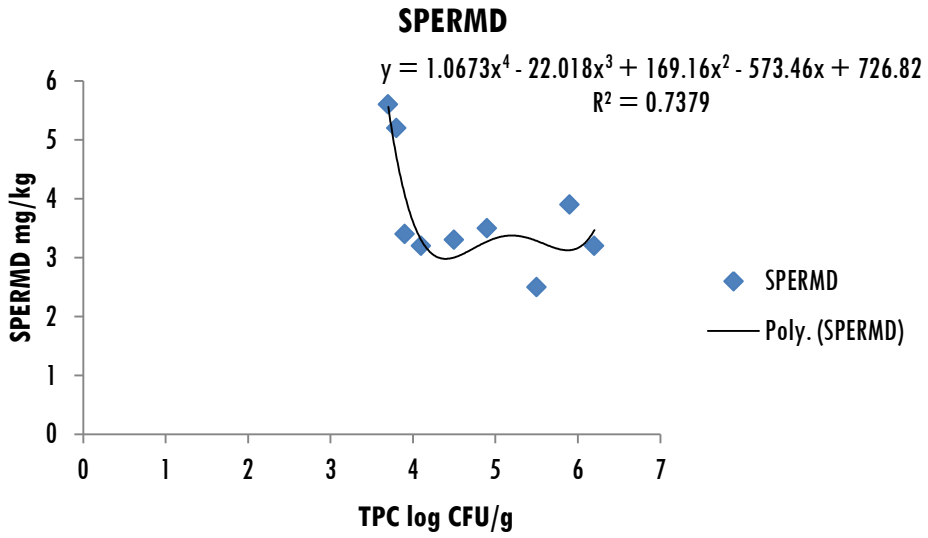
d)


TYR
y = 0.0515x⁴ - 1.0711x³ + 8.2736x² - 27.639x + 34.787
R² = 0.9839
Tyr mg/kg
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
7
TPC log CFU/g
TYR
Poly. (TYR)

e)



f)



g)

Figure: 7.1 (a-g) Correlations between the microbial flora (TPC) and the concentrations of PUT, CAD, AGM, HIS, TYR, SPERM, SPERMD in gutted *Lologo duvauceli* at 4^0C storage temperature

7.5Conclusion

The concentrations of seven biogenic amines (BA) were simultaneously determined in Indian squid (*Loligo duvauceli*) in different storage studies. The relationship between the formation of BA in Indian squid and the growth of total plate count during storage was also investigated. Results showed that putrescine, cadaverine, histamine and tyramine were the dominant BA in the studied samples, but the concentrations of histamine and tyramine were mostly less than 50 and 100 mg $kg^{-}1$, respectively. The growth of bacteria (TPC) in squid strongly and positively correlated with the formation of amines (such as putrescine, cadaverine, histamine and tyramine) during storage, except for spermine, spermidine and agmatine. Based on the study BA index limit of acceptability may be established in a range of 150-230mg/Kg in Indian squid in correlation with the total plate count.

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Chapter

8

CONCLUSION AND RECOMMENDATION

Fresh squid is a highly perishable product and spoilage of squid affects the its quality and hence its status as a commodity of commercial importance. Understanding the shelf-life of the product in relation to time – temperature relation is of significance as the product is associated with the development of toxic chemicals in the system due to the biochemical process occurring post mortem. This study was undertaken to understand the effect of temperature and storage conditions on the shelf-life with special reference to the toxic chemical viz., the biogenic amines accumulation and the possible mitigation measure to address the very important food safety hazard.

The thesis on the topic entitled "Evaluation of Biogenic Amine as a quality index in Indian Squid (*Loligo duvauceli*)" is presented in 8 chapters.

The **chapter I** gives a brief account of the present fisheries production, export of sea foods and the significance of squid in the export market. The chapter explains the significance of research work undertaken and the objectives focused with that aim.

The **chapter 2** details the current scientific literature regarding biogenic amine. Biogenic amines represent a group of low molecular mass organic compounds occurring in all organisms. Enzymatic decarboxylation of free amino acids and other metabolic processes lead to the production of BAs in food products. These BAs are produced by bacterial decarboxylation of amino acids and any foodstuffs exposed to microbial contamination during processing or storage may accumulate BAs. The concentration of BAs like

histamine, tyramine, cadaverine, putrescine, agmatine and spermidine gives therefore a good indication of the freshness of foods. The determination of biogenic amines in non-fermented or fresh and processed foods is of great interest and great value not only due to their toxicity but also because they can provide a much useful information on the food safety issues besides useful as an index of spoilage. For these reasons, it is important to monitor the levels of BAs in foodstuffs and hence the study assumes significance.

Proximate composition of Indian squid, *Loligo duavauceli,* demonstrated as a species rich in key nutritional elements required by the human nutrition is discussed in **Chapter 3**. The study shows squid contain significantly valuable sources of protein with the more prominent levels of essential/non– essential amino acid ratio. Besides significant levels of C_{20-22} long chain n–3 polyunsaturated fatty acids, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and greater n–3/n–6 fatty acid proportion demonstrated that these cephalopods are good sources of well–balanced diets. The study also showed that iced stored squid cause leaching of essential components mainly proteins responsible for the good organoleptic and nutritional characteristics. The extent of leaching affects the overall quality of the squid. It is, therefore, recommended to avoid direct contact with ice when transported from landing centers to domestic market or to processing plant.

The **Chapter 4** provide information on the quality markers in Indian squid (*Loligo duvauceli*) based on biogenic amine. The result shows that chemical and physical conditions of squid play vital role in defining the quality of the product. Since the spoilage of squid starts immediately after the catch which largely dependent on the temperature prevailing and gut contents at death; sooner the squid cooled the better will be the quality and shelf life. The results of the study indicated that the gutted squid (*Loligo duvauceli*)

stored in ice had longer shelf life and better quality when compared to the whole squid. TMA and TVBN, the chemical indices traditionally used, showed an increase during the days of storage in whole squid while a slow increase was noticed in gutted squid, serving a little to estimate squid freshness and remaining shelf life. Biogenic amines especially PUT, CAD, AGM, TYR and HIS, on the contrary, could be useful freshness indices for *Loligo duvauceli*, because their contents progressively increased from the beginning of storage in both treatments. Whole and gutted squid sample in ice storage, the level of histamine and tyramine did not exceed limits and has no adverse health effects. But there was a significant correlation between the treatment, storage time and amine content (histamine, putrescine, tyramine, agmatine and cadaverine) in squid samples, indicating the possibility of using them as quality makers, spoilage makers in squid. The chapter concluded with the finding that the squid (*loligo duvauceli*), whole and gutted, could be stored on ice in good acceptable condition up to 10 and 12 days respectively.

In **Chapter 5,** the quality of the squid as function of different storage conditions viz., -18±2°C, 0±2°C, 4±2°C and 30±2°C was evaluated to identify the appropriate quality of the squid. The study revealed that with increase in storage period and temperature, decompositions occurred by biochemical and bacteriological interaction. The storage temperature of -18±2°C and 0±2°C prevent the growth of bacteria which slowed down the bacterial proliferation and reduced the decomposition in squid and hence reduced production of biogenic amines, while storage temperatures of 30±2°C allowed a bacterial proliferation at a faster rate which reduced the shelf-life of stored squid or accelerated its spoilage. Therefore, it is suggested that chilling squid rapidly after catch (≤4°C) is the most ideal treatment to extend the shelf-life of squid. As far as quality indices is concerned, the chemical indicators such as the

concentration of putrescine, cadaverine, agmatine, histamine and tyramine show a better correlation with the organoleptical and microbiological indicator to determine the quality and freshness of squid and to detect the squid spoilage.

In commercial practice, often absolute conditions are abused leading to quality losses. **Chapter 6** discusses the effect of delayed icing on squid using chemical (BA, TMA, TVBN & Ph), microbial (TPC & PC) and sensory methods. The BA study showed that there is no important risk of HIS and TYR as the more dangerous amines in food because their levels were under the limit and negligible over the period. However, all other BAs (PUT, CAD, AGM) increased during the storage, the values of PUT and CAD was clearer than the others indicating that these amines can be good markers to evaluate squid quality. It is indicated that the temperature and the duration of storage after catch significantly affected on the levels of BAs and generally the levels in samples with 8 h delay before icing was significantly higher. TMA, TVBN sensory evaluation and microbial counts, as in other related works, can still be considered useful additional data to follow the degradation process. The study showed that chemical especially BAs and microbial changes of squid correlated well with sensory qualities and demonstrated that immediate icing is the important solution for maintaining the quality and shelf life of Indian squid (*Loligo duvauceli*).

Chapter 7 discusses the biogenic amine production in different storage conditions. The concentrations of seven biogenic amines (BA) were simultaneously determined in Indian squid (*Loligo duvauceli*) in different storage studies. The relationship between the formation of BA in Indian squid and the growth of total plate count during storage was also investigated. The growth of bacteria (TPC) in squid strongly and positively correlated with the

formation of amines (such as putrescine, cadaverine, histamine and tyramine) during storage, except for spermine, spermidine and agmatine. Although all biogenic amines were less abundant than in pelagic fish, they may also be used as indicators of freshness and/or spoilage in squid. However, the biogenic amine index, which considers cadaverine, putrescine, histamine, and tyramine, has several advantages as an indicator of squid quality. Taking into account sensory and microbial data, an acceptability limit of the biogenic amine index could be established in 150-230 mg/Kg.

Recommendations

- Proper evisceration of the squid would help to bring down the biogenic amine production during storage and extend the shelf life of the product. Hence it is recommended to popularize the practice of proper pre-processing and handling to produce safe and quality squid to the consumer.
- The presence of the biogenic amines, produced by the microbiological spoilage of squid could be suggested as quality indicator in squid, *Loligo duvauceli.*
- The usefulness of biogenic amines as quality index depends on the condition of the squid, the levels of precursor free amino acids and conditions of storage and need to be considered while addressing the quality of the squid.
- Among the BA cadaverine, and putrescine in squid were the most obviously changed amines during the storage at different temperatures, and these biogenic amines could be used as effective quality indicators for the freshness of the squid.

- Biogenic amines are heat stable compounds, cooking or prolonged exposure to heat does not eliminate the toxin. Thus, biogenic amines could be effectively used as quality index in the squid even after heat treatment.
- A sum of putrescine, histamine, tyramine and cadaverine can be used as an index of quality in squid because their concentrations increase during storage and correlate well with the microbial load and sensory evaluation.
- The quality index developed could be effectively used for evaluating the quality of squid in commercial operations.

The risk of histamine poisoning could be effectively controlled by following the principles of good manufacturing practices and Hazard Analysis Critical Control Point (HACCP) system appropriate to fish and fishery products. Maintaining the commodity under controlled temperature regime by appropriate icing practices, hygiene handling and transportation under controlled conditions are to be strictly adhered to in order to avoid the onset of histamine issues.

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LIST OF PUBLICATIONS/PRESENTATIONS

- Anju K A and Sankar T.V. (2017). Effect of Packaging in Biogenic Amine Production in Indian Squid (*Loligo duvauceli*) stored in iced condition. International Journal of Science and Research 6 (10), 225-229.
- K. B. Biji, K. R. Remya Kumari, K. A. Anju, Suseela Mathew and C. N. Ravishankar. (2016). Quality characteristics of yellowfin Tuna (*Thunnus albacores)* in the fish landing centre at Cochin, India. Fishery technology 53(2016): 313-319.
- Paper presented on 'Effect of storage condition on Biogenic amine production in Squid (*Loligo duvauceli*)' in 26th Swadeshi Science Congress, National Seminar at Central Marine Fisheries Research Institute, on 7 to 9 November 2016, Kochi; Anju K.A and T.V.Sankar; Central Institute of Fisheries Technology, Kochi
- Paper presented on 'Assessment of freshness of squid (*Loligo duvauceli*)' under chilled condition using a quality index scheme, in International Conference on Science and Technology for national development, at Kerala University of fisheries and Ocean Studies, Panangad, Kochi; on 25 to 26 October 2016, Anju K.A and T.V.Sankar; Central Institute of Fisheries Technology, Kochi

ANNEXURE

QIM scheme for whole raw squid stored in crushed ice

	Freshness quality parameters	Description	QIM score			
	Quality attributes		**A**	**B**	**C**	**D**
1	**Skin (dorsal side)**					
	Appearance/colour	Very bright, well defined pigments of different sizes and colours (brown, purple, rose and dark red), iridescent skin	0	0	0	0
		Bright, becoming discoloured	1	1	1	1
		Rather dull, without shine, purplish in the central axis of the body, general orange/pink areas	2	2	2	2
	Odour	Seaweedy, (sea) fresh	0	0	0	0
		Slightly seaweedy	1	1	1	1
		Neutral, slightly fishy	2	2	2	2
		Intense, metallic, fishy	3	3	3	3
	Mucus	Transparent, watery, shining	0	0	0	0
		Slightly milky, moderate or absent	1	1	1	1
2	**Flesh**					
	Texture	Firm, tense, consistent	0	0	0	0
		Soft, less consistent	1	1	1	1
		Flaccid, flabby	2	2	2	2
3	**Eyes**					
	Shape/appearance	Convex	0	0	0	0
		Flat	1	1	1	1
		Concave	2	2	2	2
	Ocular tissue	Translucent, watery	0	0	0	0
		Slightly opalescent	1	1	1	1
		Opalescent	2	2	2	2
4	**Mouth region**					
	Odour	Seaweed, fresh	0	0	0	0
		Neutral	1	1	1	1
		Slightly fishy	2	2	2	2
		Intense, fishy, acid	3	3	3	3
	Mucus	Absent or clear transparent	0	0	0	0
		Slightly yellowish	1	1	1	1
	Range of QIM score		**0–16**	**0–16**	**0–16**	**0–16**

Date:
Time:

Name & Signature