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Training manual on
QUALITY ASSURANCE OF FISH AND FISH PRODUCTS
(Under ICAR-CIFT SCSP Component)
20-24th November 2023
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SPOILAGE MECHANISM AND QUALITY CHANGES IN SEAFOOD

Viji P. and **Jesmi Debbarma**

Fish is an important food item for mankind in view of its high protein content, vitamins and poly unsaturated fatty acids. However, the seafood quality is unique and compared to terrestrial meat, seafood deteriorates rapidly post-mortem as a consequence of various biochemical, autolytic and microbial breakdown mechanisms.The high ambient temperature of our country favours the rapid growth of micro-organisms. With higher ambient temperatures, fish quality deteriorates very rapidly. Spoilage of fishoccurs concurrently and independently, their relative importance varying with species of fish, environmental conditions, method of slaughter and post-mortem handling, storage procedures and processing conditions.

Post-mortem changes in fish

After the fish dies, the supply of oxygen to fish muscle ceases and thus the synthesis of adenosine tri phosphate (ATP) stops. The glycogen reserves in the body convert to lactic acid anaerobically instead of CO_2 and water, leading to a reduction in muscle pH. At this stage, the breakdown of Adinosine Tri Phosphate (ATP) begins. As pH decreases, muscle proteins approach their iso-electric point and denaturation begins. The decline in pH affects the quality of fish muscle in that the flesh becomes firmer and the tendency to drip is enhanced (Connel, 1975). Anaerobic catabolic activity continues till the ATP level reduces up to 5% in the muscle. Drop in ATP level initiates a combination of actin and myosin leading to the formation of actomyosin. This results in a rigid condition of the muscle, called rigor mortis. Within hours, depending upon species, condition and temperature, the muscles begin to contract and the fish enter into the stage of stiffening or rigor mortis.

The development of rigor is closely related to temperature. The length of time between death and onset of rigor is determined by the relative activities of enzyme systems responsible for ATP degradation. This in turn is controlled by the relative

concentrations of ATP, creatine phosphate and glycogen in the muscle tissue at the time of death.

The ATP is broken down to adenosine diphosphate (ADP), adenosine monophospahte (AMP), inosine (IMP) and then hypoxanthine (Hx). The initial sweet, meaty and species characteristic flavours of fresh fish reflect the combination of IMP and free amino acids present in the flesh, as well as some sugars and sugar phosphates (Jones, 1961).

Spoilage of fish

Spoilage of fish begins as soon as the fish dies. In tropical countries, spoilage begins rapidly within few houres of landing, if the fish is not properly cooled. In raw fish, spoilage takes place mainly due to 3 reasons ie., enzymatic, microbial and chemical action.

1. Enzymatic spoilage

After death, anaerobic glycolysis takes palce and glycogen will converted to lactic acid. The accumulation of lactic acid in the muscle brings down the pH to 6.2-6.5. The decline in pH accompanied by natural post mortem stiffening is called rigor-mortis. After rigor mortis, the muscle enzymes begins hydrolysing nucleotides, protein and lipids in the fish muscle. Enzymatic hydrolysis result in the development of compounds with undesirable flavour and odour. In heavly fed fishes, autolysis leads to belly bursting due to higher rate of hydrolysis by muscle enzymes in the belly area.

2. Microbial spoilage

Enzymatic hydrolysis degrades the protein into peptides, peptones and aminoacids, which together with vitamins, minerals and nucleotides constitute an ideal medium for microbial proliferation. Fish spoils mainly due to the action of bacteria. The saprophytic bacteria (Psedomonas, Flavobacterium, Acinetobacter, Aeromonas etc.) present on the slime, gill, skin, intestine etc. invade in to the tissue and start decomposing protein, lipid etc. Microbial action in fish leads to the production of compounds like Tri Methyl Amine(TMA), ammonia, histamine, indole,hydrogen sulphide, methyl mercaptan etc. All these compounds together gives undesirable offodour and off flavour to the fish and hence the fish become unacceptable for consumption.

3. Chemical spoliage

The most important chemical spoilage takes place in fish is the fat oxidation. Fish lipid have high concentration of poly unsaturated fatty acids and hence undergoes oxidative changes. Fat oxidation give rise to problems such as rancid odour and flavour as well as discoloration. The lipid oxidation is initiated and accelarated by oxygen, heat, light, presence of metallic ions like Cu^{2+} and Fe^{2+} etc. The products of fat oxidation like carbonyls, aldehydes etc. further reacts with proteins and aminoacids and reduces its solubility.Fattyacid is oxidised to hydroperoxides initailly and the peroxides further get oxidised to aldehydes and ketones with typical rancid odour.

Changes in fish due to spoilage

Flavour changes

During spoilage many undesirable compounds are produced in the fish. Nucleotide degradation result in the production of hypoxanthene which is bitter in taste. Moreover, the accumulation of ammonia, and other voltile bases like Tri methyl Amine (TMA), formaldehyde etc. brings about definite changes in the inherent flavour of the fish.

Color changes

Color is an important factor in sea food quality. Color of the fish is changed due to enzymatic oxidation or nonenzymatic fat oxidation and pigment oxidation. The importanrt discoloration in seafood is described below.

Blue or black discoloration in shrimp and lobster

The development of black spot in shrimp and lobster is due to the presence of an enzyme polyphenol oxidase. Black spot is formed by the oxidative action of this enzyme, particularly tyrosinase on the aminoacid tyrosin, thus melanin is formed. The pigment is formed on the shell surface initially and in advanced stages, on the underlying meat. This makes the shrimp unattractive for marketing. Discoloration at the butt of the tail, black spot between the segments are pronounced in moribund lobster. In crab, in addition to black discoloration, blue discoloration also occures which is induced by the oxidation of haemocyanin pigment in the blood and is non enzymatic.

Yellowing of fish flesh

During chilled or frozen storage, some fishes developes an yellow color on the surface. This is due to the oxidation of fat as well as carotenoids. Freezing or other process desrupt chromatophores and release carotenoids and their migration to subcutaneous fat layer causes.

Brown discoloration

This type of discoloration is more pronounced in dark fleshed fishes such as tuna and mackerel. Myoglobin in the blood get oxidised to metmyoglobin and color of muscle changes to brown. Reaction between protein or aminoacid with the product of lipid oxidation also lead to brown discoloration.

Changes in texture

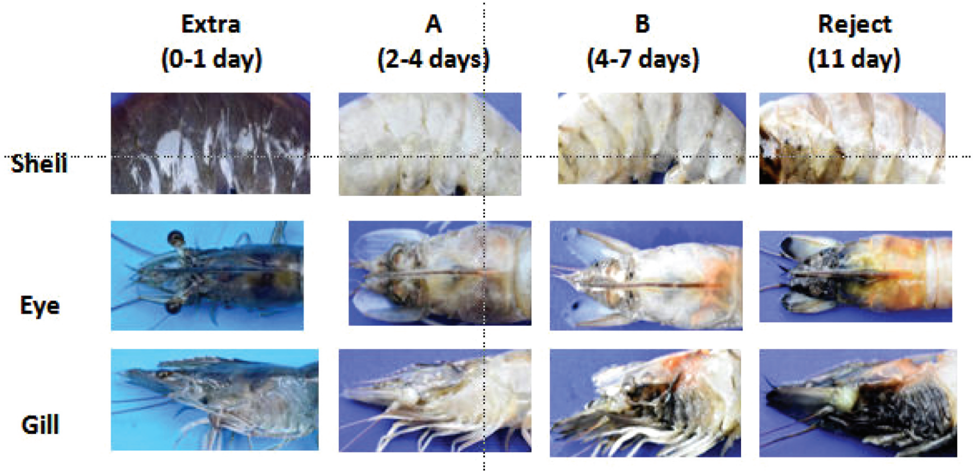
The collagen fibrils attaching the muscle bundlesmaintain the toughness and integrity of fish muscle. The action of microbial as well as autolytic enzymes on fish flesh brings about undesirable changes in the texture. The enzymes degrades muscle proteins and breake down the connective tissue in muscle. As a result, the texture of fish muscle become soft. Degradation of myofibrillar proteins also contributes to softenss of muscle during storage. These undesirable changesare due to the activity of autolytic enzymes (e.g. collagenase,ATPase) degrading different proteins and breaking down theconnective tissue in muscle meanwhile the spoilage microorganisms multiply rapidly and promote the progress of spoilage.The fish muscle loses its stiffness, elasticity, springines and chewiness after spoilage.

Belly bursting

Enzymatic spoilage causes belly bursting especially during a period of heavy food intake. Heavly fed fish have a high content of digestive enzyme in the intestinal tract. In such fish, autolysis begins rapidly and in the dissolved gut component, bacteria proliferates and produces gases such as carbon dioxide and hydrogen sulphide. This gas production lead to belly bursting after short storage period.

Days	**0 day**	**5th day**	**15th day**
Eye	Convex with transparent cornea, black pupil, bright yellow sclera	Convex, slightly opaque cornea, black but dull pupil, Reddish sclera with yellow tinge.	Concave eye , red cornea, opaque and red pupil, ingression of blood, red sclera
Gills	Bright blood red gills covered with thin layer of transparent mucous	Dull red color with bleaching from the dorsal gill rays	Pale brown color, all gill rays bleached

Changes in grouper during iced storage



Changes in *Penaeus vannamei* (Pacific white shrimp) iced storage

Factors affecting spoilage of fish

Temperature

It is well known that both enzymatic and microbiological activity is greatly influenced by temperature. However, in the temperature range from 0 to 25°C, microbiological activity is relatively more important, and temperature changes have a greater impact on microbiological growth than on enzymatic activity. In general, an increase of 5^0C temperature will double the rate of spoilage. Many bacteria are unable to grow at temperatures below 10°C and even psychrotrophic organisms grow very slowly, and sometimes with extended lag phases, when temperatures approach 0°C. The shelf life of fish products, therefore, is markedly extended when products are stored at low temperatures. High temperatures are partly responsible for the speed of the oxidation processes. In addition, direct sunlight, wind, heat, light (especially UV-light) and several organic and inorganic substances may also accelerate oxidative processes. It is often critical to reach the desired short-term storage temperature rapidly to maintain the highest visual quality, flavour, texture, and nutritional content of fresh fish.

Intrinsic factors

The spoilage rate of fish is affected by many parameters like species, pH, fishing ground, season etc. and the fish spoil at different rates depending on these factors. In general it can be stated that larger fish spoil more slowly than small fish, flat fish keep better than round fish, lean fish keep longer than fatty fish under aerobic storage and bony fish are edible longer than cartilaginous fish. Several factors probably contribute to these differences and whereas some are clear, many are still on the level of hypotheses.

Factors affecting spoilage rate	**Relative spoilage rate**	
	Fast	**Slow**
Size	Small fish	Larger fish
post mortem pH	High pH	Low pH
Fat content	Fatty species	Lean species
Skin properties	Thin skin	Thick skin

Handling

Rough handling will result in a faster spoilage rate. This is due to the physical damage to the fish, resulting in easy access for enzymes and spoilage bacteria. Physical mishandling in the net, such as very large catches, fishermen stepping on fish or throwing boxes, containers and other items on fish catch, may cause bruises and rupture of blood vessels that can accelerate the rate of spoilage. When fish is in rigormortis, rough handling can cause gaping. Apart from the microorganisms that fishes have at the time of capture, more is added via unhygienic practices and contaminated equipments, utensils and storage containers.

Initial bacterial load

The microflora on tropical fish often carries a slightly higher load of Gram-positives and enteric bacteria but otherwise is similar to the flora on temperate-water fish. Basically, bacteria populations on temperate fish are predominantly psychrotrophic reflecting water temperatures of about 10^0C while fish from the tropics have largely mesophilic bacteria.

Methods of capture

The fishing gear and method employed determines the time taken between capture and death. Fish caught in gillnets struggle much to escape and hence, they are bruised by the net which increases exposure to microbial growth and subsequent deterioration. Fish caught by hook and line methods, on the other hand, die relatively quickly and therefore bruises and stresses are likely to be minimal. Physical mishandling in the net due to long trawling nets and very large catches accelerates spoilage. The large catches in the net are compacted against each other resulting in the fish getting bruised and crushed (especially small sized fish) by the heavy trawl net.

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QUALITY ISSUES IN SHRIMP PROCESSING

Viji P., Jesmi Debbarma, Madhusudana Rao. B. and **Ahamed Basha K.**

The evaluation of the general quality and shelf life of fish and fish products is based on organoleptic, chemical and microbiological tests. However, in quality evaluation of crustaceans, particularly shrimps, there are certain specific spoilage problems mostly associated with the high free aminoacid content of shellfishes. Shrimp spoils rapidly compared to fishes on account of its neutral pH, high content of moisture, free amino acids and nitrogenouscompounds. Itswholesomeness under iced storage isaffected by chemical changes, bacterial growth and melanosis. The major problems associated with shrimp processing is black spot formation or melanosis and indole formation.

Melanosis

The darkening of pigments in membranes and meat just under the shrimp shell (melanosis) is commonly known as black spot. Blackspot formation in shrimp is harmless but objectionable discoloration occurring primarily along the swimmerets, head, tail and nearby shell areas reduces the consumer's appeal over the product. Blackspot is caused by a system of enzymes known as polyphenoloxidase (PPO) that are naturally present in the shrimp. PPO oxidizes phenols to quinones and auto-oxidation of the quinines in turn giving rise to dark pigments of melanin. Its intensity varies among species as it is dependent on substrate and enzyme concentration.

Melanosis has no impact on the flavor of shrimp and is not harmful to consumers. However, the black spots can drastically affect consumer acceptability of the products and significantly diminish their market value. Since blackspot is caused by enzymes naturally present in the shrimp, the darkening occurs before bacteria grow and causes deterioration and spoilage.

Control of Melanosis

None of the acceptable processing techniques are totally efficient at controlling melanosis. Good harvest practices along with a strict control of the cold chain is

found to be effective in slowing down enzymatic reactions, but can necessarily does not stop the reaction. Hence, it is very important to avoid every chances of occurrence; because once melanosis starts, it is irreversible.

Frozen storage can effectively retard physicochemical changes of shrimp, however, black spot formation (melanosis) can take place after thawing. One method to retard melanosis is by keeping the material completely immersed in a medium of ice and chilled water with a distinct layer of water above the material. Beheading of shrimp immediately after harvesting and subsequent storage in ice also reduces the black spot formation as the enzyme is concentrated in the head region. However, after removing the head, care should be taken to thoroughly wash the tails to eliminate the enzyme that can promote melanosis.

Chemical treatment

Several melanosis inhibitors including sulfiting agent and 4-hexylresorcinol have been used in conjunction with iced storage.4-hexylresorcinol blocks the enzyme responsible for black spot and maintains a GRAS (generally regarded as safe) status; however, the compound is currently not authorized in the E.U countries. Metabisulfite, a relatively inexpensive inorganic chemical that has been used for more than 40 years. Metabisulfite acts with the intermediate reaction elements of melanosis, in particular quinone and sulfaquinone, by reducing the oxygen available for oxidation reactions. However, a small percentage of consumers are allergic to sulfites. Also if this chemical is used in excess hydrogen sulfide gas will be produced, which is very poisonous, and presents a serious health risk for people entering the hold for unloading. Hence, USFDA has recommends every sulfited and packaged shrimp products should include a statement that the product contains sulfites. Metabisulphites have been in use for many years to control melanosis development. However, metabisuphitesare treated as allergens and the European Union demands labeling on shrimp packages where sulphite residue exceeds 10 ppm (Mark Edmonds, 2006).

Antioxidants

Since oxygen is needed for PPO activity, removal of oxygen will help in reducing the black spot formation**.** Reducing agents like ascorbate, erythrobate and reducing sugars will not only utilize the sugar but also converts quinines to back to di-

phenols thus reducing the progress of melanosis. These treatments can temporarily retard the black spot formation, however, black color returns after thawing.

The maximum black spot permitted is 10% by count in shell-on types and 5% by count in peeled type.

Metabisulphite dip treatment schedule for shrimp

As there is much variation in the general harvest technology (net harvesting, mechanical harvesting etc.) and product form (head-on, headless etc) the treatment schedule and the concentration of metabisulfite may also vary and a generalised post harvest treatment can not be suggested. Also, there exists a wide variation in PPO activity between different species, and different body parts. USFDA and codex Alimentarius suggest a 1 min dip in a 1.25 % solution of sodium bisulphite as a part of good manufacturing practice for prawn or shrimps. However, this schedule may vary depending on the species and importing country's specifications.

The following practice may be followed during after the harvest of shrimp in order to reduce balck spot incidence

1. Dip the shrimp immediately after harvest in clean potable chilled water.
2. Immerse the baskets of shrimp for 1 minutes in a 1.25 percent solution of sodium metabisulfite (100 l of 1.25 % solution of sodium bisulphate is sufficient to treat 200kg fish)
3. Immediately after the treatment, store the shrimp in ice or ice water for transportation to the processing facility.

If the harvested shrimps have to be stored forprolonged time in ice, an additional treatment is necessary during storage. This is because, the chemical may slowly leach out of shrimp during storage, and melanosis may onset due to the deficit of chemical. Likewise, if the shrimp need to be transported farm to processing facility in immersed condition, and if takes a longer time, then the chemical concentration should be reduced to 0.25% (w/v). Hence the treatment schedule has to be ultimately charted out considering the condition and duration of storage and transportation.

Residual sulphite content in processed shrimp

Because some consumers may be sensitive to sulfating agents, the FDA has established a regulatory limit of 100 ppm for sulfite residue on shrimp. This level is adequate to prevent melanosis. Other countries have limits ranging from 60 to 100 ppm on raw shrimp and as low as 30 ppm for cooked products. Japan accepts a maximum of 100 ppm sulphite calculated as SO_2 residue in raw shrimp flesh and 30 ppm in cooked meat, while in the European Union, the rate varies according to the size of shrimp.

Indole formation in shellfishes

Indole formation depends exclusively on development of bacteria producing enzymes called tryptophanases, that oxidize the free L-tryptophan present in muscle tissues producing indole, skatole (methyl indole) and indolacetate. Several bacteria genera have species that are indole positive, particularly *Proteus, Escherichia, Edwardsiella*, *Flavobacterium, Aeromonas, Plesiomonas, Bacillus*, etc. Indole detection has long been used as an indicator of shrimp spoilage. Based on data collected by the Food and Drug Administration-FDA/USA, indole levels in fresh shrimps are lower than 1 mg/100g and there is a good correlation between indole concentration and organoleptic evaluation. According to these studies, shrimps are classified in class 1,when they do not show any organoleptic evidence of spoilage; when spoilage is first detected, they go to class 2, and when spoilage is clearly defined they are classified in class 3; the corresponding indole levels are < 25 µg/100g for class 1, and > 25 µg/100g for classes 2 and 3. Indole levels have been used to confirm the sensory evaluation of shrimp decomposition and a limit of 25 µg/100g has been used in several countries to differentiate passable shrimp from shrimp in the first stage of decomposition the indole could be considered a useful indicator in assessing the history of shrimp if high temperature is suspected or bad hygiene conditions have been applied.

Veterinary residues in farmed shrimp

It is a common practice to use antibiotics (medicinal drugs) in shrimp farms to control the outbreak of diseases. Excess use of antimicrobials, contaminated feed and pesticide residue in water can cause high levels of contaminants in harvested shrimp. Presence of chemical residues in shrimp leads to rejection of the product

by the exporting countries. Misuse, overuse and improper withdrawal periods of antimicrobials could result in harvested products containing certain chemicals above the established maximum residue levels (MRL). These malpractice can also lead to the development and propagation of antimicrobial resistance (AMR) along the food chain which could result in death of 10 million people annually by 2050. As these products pose a health risk to consumer, they are not allowed on the market. Residues of veterinary medicinal products in food are potentially harmful to the consumers eg., chloramphenicol causes aplastic anaemia and nitrofurans are carcinogenic. Nitrofurans are a group of synthetic antibiotics that were chemically derived from furans and contain a characteristic 5-nitrofuran ring. Nitrofurans include nitro-furazone, nitrofurantoin, furaltadone and furazolidone. Nitrofuran parent compound metabolise rapidly after ingestion by the shrimp to form currosponding tissue bound metabolites. These metabolites will bind to the shrimp tissue proteins fro many weks after treatment. Nitrofuran metabolites are stable during storage and are not destroyed by cooking, frying, grilling, roasting and microwaving of meat. The EU has established a minimum required performance limit (MRPL) of 1 μg/kg (1 ppb) for nitrofuran metabolites and 0.3 μg/kg for chloramphenicol in aquaculture products (E U, 2003). Prudent use of antibiotics, proper regulation on the usage of antimicrobials, surveillance of AMR in bacterial pathogens in aquatic food products, creating awareness on the implications of the antibiotics in aquaculture will curtail the AMR and antibiotic residues in aquaculture environment.

GOOD HYGIENIC HANDLING PRACITCES FOR ENSURING THE SEAFOOD QUALITY

Viji P. and **Madhusudana Rao B.**

Seafood is highly perishable on account of its higher moisture content and soft texture and the maintenance of fish quality is difficult than in the case of meat products. Hygienic handling is one among the important extrinsic factors that influence the quality of fish. The fish and fishery products intended for sale in the retail market must be safe. They should not cause any illness or injury to the consumer. For the production of safe fish, not only the quality of the raw material (such as fish, shrimp) but also the quality of water, ice, ingredients (such as salt) and the cleanliness of food contact surfaces (such as tables, utensils) and personal hygiene of the workers is very important. Using fresh raw material and good quality ingredients helps in producing fishery products that meets the regulatory requirements of Food Safety Standards Authority of India (FSSAI).

Various handling activities including fish landing, washing, dressing, packing, distribution, distribution and selling take places in retail fish markets which invites many risk factors creating additional sources of bacteria and contamination. Therefore, the implementation of hygienic measures is recommended to prevent contamination of fish at retail markets. Good handling always results in economic benefit to the farmers, processors and retailers because the consumers are willing to pay more for a premium quality product. Implementing hygienic handling practices helps to reduce microbial growth and delay spoilage. Hygienic handling practices ensure the supply of safe and quality fish to consumers while meeting the standards of national and international food safety regulatory bodies.

Onboard handling practices

Fish and fish products intended for human consumption should be handled properly to prevent contamination and spoilage till it reaches the end user. Poor onboard handling practices damages the fish and speed up the spoilage process resulting in post-harvest losses. The important points are

- The fishing vessel and equipment should be thoroughly cleaned using clean water before and after every fishing trip.
- Fishing vessel must be kept free of pests using pest control devices.
- Sea water from fishing harbor/landing centre should never be used for cleaning. Only tap water from the public water supply, clean well / borehole water that has been treated with chlorine or clean seawater should be used to clean boats and equipment.
- All fish contact surfaces should be made of non-toxic, smooth materials to minimize the build-up of slime, blood, scales etc. from the harvested fish to reduce the risk of microbial contamination.
- Handling areas should not have sharp corners and projections to avoid physical damage to the fish.
- Fish should be washed well with potable water/ clean sea water to remove dirt and other foreign matter if any, immediately after harvesting.
- In aquaculture site, the harvested fish should not be dropped on to muddy floor or hardy surfaces to prevent contamination and physical damages, respectively.
- Fish should be kept away from objectionable substances such as grease, fuel oil, drainage, bilge water, smoke, and other solid or semi-solid to prevent contamination.
- Bruised, damaged and decomposed fish shall be separated from the catch during sorting.
- Bleeding of fish if any, should be carried out as early as possible.
- Mishandling of the fish such as throwing, standing on fish, exposing to sunlight etc. needs to be strictly avoided.
- Fish should not be exposed to sunlight for a longer duration as it causes dehydration and accelerate spoilage.
- The condition of the equipment and utensils should be such that it minimizes the build-up of residues and prevents them becoming a source of contamination.

Chilling and Storage

The ambient temperature of tropical countries favours the growth of mesophilic bacteria. A 5°C rise in temperature doubles the rate of spoilage. Hence, the temperature of fish should be immediately brought down to near 0°C after harvesting to delay the microbial growth and spoilage. Time and temperature control during post-harvest stage are the most effective tools to ensure food safety.

- Store the harvested fish in ice or chilled/refrigerated seawater as early as possible to bring down the core temperature as close to 0°C avoid bacterial spoilage.
- Fish and ice should be tightly packed in the container in shallow layers (1:1 fish to ice ratio) to avoid free space which otherwise cause faster melting of ice.
- In refrigerated or chilled sea water systems, care should be taken to control overloading of fish to prevent physical damage.
- Boxing/shelving storage areas of fishing vessel should apply minimum pressure on the fish.
- Clean and chemical-free ice made from potable water should be used and it should be protected from contamination.
- Fish harvested at different times should be stored separately as they are in different stages of spoilage.
- Use of crushed ice with sharp edges must be avoided as it causes physical damage to the fish.
- The containers used for storage should be designed to provide adequate drainage and should ensure proper cleaning and disinfection to avoid contamination.
- The boxes used for storage should not be over filled or stacked too deep as it exerts pressure on the fish.
- Fish room/hold must be strong, corrosion resistant, insulated, and easy to clean with smooth surfaces and allows adequate drainage for melt water.

Handling at landing centre

A great deal of fish handling occurs at landing centers or harbours. It should be ensured that the fish leaving the fish landingcentres is of an assured quality and safe for human consumption. Proper handling practices assure the quality of fish and reduce post harvest loss at landing centres.

- The ice should never be dragged on the floor and must be stored in clean containers.
- Never use seawater from landing centre for cleaning the fish.
- Do not throw the fish on hard surfaces to prevent physical damage and contamination.
- Sorting the catch on beaches should be avoided.
- All the containers/contact surfaces used for unloading and weighing shall be cleaned & disinfected immediately.
- Entry of flies, cats, dogs, rodentsetc. in the fish handling premises may be prevented.
- Adequate supply of clean, potable water should be ensured at the landing centres.
- Fish wastes and offal shall be separate boxes with tight lids and shall be discarded properly.

Handling at retail markets

Various handling activities including fish landing, washing, dressing, packing, distribution, distribution and selling takes places in retail fish markets which invites many risk factors creating additional sources of bacteria and contamination. Therefore, implementation of hygienic measures is recommended to prevent contamination of fish at retail markets.

- Location of retail fish market should be away from vegetable market, meat or other food markets.
- Facilities for potable water, electricity and proper hygeinic sewage disposal should be provided.

- Facilities like toilet and arrangement for washing of hands should be provided near the market premises.
- Selling / Auction Platform/ tables should be elevated with smooth vitrified tiles with side protection and drain pipe.
- Cutting and filleting of fish to be separated from selling area to prevent cross contamination.
- Separate area for crate and utensil washing must be provided.
- Waste materials should be properly segregated, iced and stored in tight containers.
- Drain pipe from display tables and cutting platforms should be directly connected to main drain to avoid splashing of water
- All fish contact surfaces should be smooth, water resistant and non-corrosive.
- Effective cleaning procedure and regular cleaning schedule should be maintained.
- All the utensils should be washed within 2 hrs of use apart from daily washing at the start and end of sale.
- Utensils must be stored upside down so that they can adequately drain.
- Protection from adulteration with lubricants, fuel, pesticides, cleaning compounds, sanitizing agents, condensate, and other chemical, physical, and biological contaminants.

Hygienic fish handling in processing units

Processing units aims towards value addition of the fish thus improving the market value of the products. Hygiene and sanitation are one among the pre-requisite programmes for implementing HACCP in seafood processing units. The following important measures need to be takes care of in processing units for the supply of safe products.

- Design and layout comprising sufficient working space under adequate hygienic conditions, area for machinery, equipment & storage, separation

of operations preventing cross-contamination, adequate natural or artificial lighting, ventilation and protection against pests.

- All food contact surfaces shall be smooth, durable, non-absorbent type, easy to maintain and clean and non-toxic.
- Availability of uninterrupted supply of potable water throughout for all processing operations.
- Availability of suitable facilities for temperature, humidity and other controls.
- All pre-processing and processing activities should be scheduled under HACCP system with proper documentation.
- Regular monitoring of processing unit for plant sanitation with an in-house laboratory and an in-process product quality check.
- Effective maintenance and sanitation systems including cleaning and sanitation procedures, pest control systems, waste management and monitoring effectiveness.
- All fish handlers should follow the recommended hygienic handling practices such as periodic medical examinations, regular cleaning and disinfection procedures prior and post to processing activities.

Quality of water: Water is needed for ice production, fish processing, and washing purposes. The water used for processing of fish must be of potable quality. The water used in fish processing plants must meet the regulatory standards. Seawater from coastal areas should not be used for fish handling purpose.

Quality of Ice: The ice used for chilling of fish must be prepared from potable water. The block ice should be prepared in rust free containers. Ice blocks should not be dragged on the floor. The ice blocks must be crushed with rust free equipment. Ice should not be put on dirty floor but should be placed in clean crates. Fish should be iced in 1:1 ratio of fish to ice in insulated containers. Melted ice should be replaced with fresh ice during storage.

Quality of Salt: The salt used for salting of fish should be free from foreign matter with no visible signs of dirt, oil, bilge or other extraneous matter and should not have any objectionable odour.

Cleanliness of food contact surfaces: Food Contact Surfaces are those surfaces that contact human food and those surfaces from which drainage onto the food surfaces that contact the food ordinarily occurs during the normal course of operation. Eg. tables, knives, cutting boards, utensils, fish boxes, conveyor belts, ice storage bins, gloves etc.All food contact surfaces should be adequately and routinely cleaned and disinfected. Proper washing of the food contact surfaces should include scrubbing to remove biofilms and solid waste, cleaning with detergent, rinsing with potable water, application of sanitizer followed by final wash with potable water.

Personnel hygiene

There is a possibility that the people who handle the fish can introduce hazards to to the products. Hygiene and cleanliness of workers handling fish and fish product at each stage from harvesting is very crucial in determining the safety of fish. Hence, the fishers should adopt few simple hygienic actions to prevent contamination.

- No person who is suffering from, or who is a carrier of, any communicable disease or has an infected wound or open lesion should be engaged in fish handling or transportation.
- Adequate and appropriate protective clothing, face mask, head coverings and footwear should be worn during fish handling.
- Fish handling personnel should strictly avoid the objectionable practices such as smoking, spitting, chewing, sneezing or coughing to prevent contamination.
- Fish handlers should sanitize their hands regularly in Hand Dips to prevent contamination.
- The workers must wash their hands 1) Before they start handling fish or go back to handling fish after other work 2) Immediately after using the toilet 3) Immediately after smoking, coughing, sneezing, using a handkerchief or disposable tissue, eating, drinking or using tobacco or similar substances and 4) After touching their hair, scalp or a body opening.

Control of pests: Pests such as insects and rodents carry disease causing organisms. Moreover, their droppings contaminate the food. Pests have to be controlled both within the processing plants and around the processing plant. Bait stations or glue traps at different points are need to trap the rodents. The doors and windows should be tightly fit. All entrance and exit points to be fitted with air curtains and plastic strip curtains.

1. **Prevention of cross contamination:** Cross-contamination occurs due to crisscrossing or mixing of cleaned food with raw, unwashed food. This can be prevented by ensuring unidirectional flow of men and material in the fish processing plant. The raw and cooked products should be kept separate at all times, the unprocessed material must be maintained at a temperature as close of that of melting ice (less than 4p C) to avoid multiplication of bacteria. The processed fish and packaging material should be protected from condensate or other dripping liquids and from splashes of water.

2. **Proper labeling, storage and toxic compounds:** It is necessary to ensure protection of food, food packaging materials food contact surfaces from adulteration with lubricants, fuel, pesticides, cleaning compounds, sanitizing agents, other chemical, physical, and biological contaminants. So such items should be labeled properly and stored in separate rooms.

Suggested readings

Text book of Fish Processing Technology (Ed. K. Gopakumar), ICAR, NewDelhi. pp. 38-83.

Huss, H. H., 1995. Quality and quality changes in fresh fish. In: H.H. Huss (Ed), *FAO fisheries technical paper No*. 348. FAO, Rome, Italy.

Huss, H. H., Dalsgaard, D., Hansen, L., Ladefoged, H. and Pedersen, Z. L., 1974. The influence of hygiene in catch handling on the storage life of iced cod and plaice. *J. Food Sci. Tech.,* 9: 213-221.

QUALITY DEFECTS IN FROZEN FISH PRODUCTS

Viji P.

Freezing is a method of low temperature preservation of fish that can ensure a very long shelf life and also keeps the product characteristics similar to fresh fish. However, during prolonged storage, some quality problems can arise in frozen products due to the changes in protein, fat etc. The common quality defects found in frozen fish products is detailed below.

1. Dehydration and freezer burn

During cold storage, some amount of moisture escape from the fish . Sublimation of ice takes place from the surface of unpackaged or improperly packaged fish causing what is termed as freezer burn. It is evident as white patches on the frozen fish. Fish develop a chalky white,yellow or brown colour and wrinkled appearance on the surface. When thawed, such fish presents a dry and spongy texture. Temperature fluctuations in the cold store as well as higher velocity of the circulating air causes dehydration of the product. It can be minimised by use of glazes, packaging material with very low vapour permeability, maintenance of high relative humidity in the cold store and avoiding temperature fluctuations etc.

2. Gaping of fillets

On thawing, frozen fish fillet often exhibits a phenomenon in which the connective tissue fails to hold the muscle blocks together exhibiting gaps in the musculature. Weakening of connective tissue result in the muscle segments falling apart as in cooked fish. This phenominan commonly occure in fish which are well fed at the time of capture and frozen before or during rigor. The muscle contraction during freezing tend the muscle to pull apart.

3. Discoloration

Fat oxidation in the fish muscle during storage leads to yellow discoloration. Some fish like tuna exhibit green or brown discoloration after cooking. This occurs due to the oxidation of myglobin pigment to metmyoglobin when the fish is exposed to oxidative condition during and after cooking. Loss of characteristic

pink color in lobster and shrimp is the direct consequence of the changes in the pigments like bete carotene and astaxanthene. Brown or black discoloration appear at the cut end of lobster tail during storage probably by the reactio of enzymes. The imporatnt problem in frozen squid and cuttle fish is the yellow discoloration of tubes and fillets. Treatment with a mixture of salt and citric acid is found to improve the color.

4. Toughness

Freezing and prolonged cold storage result in toughness of the meat as well as dryness on cooking. Increase in toughness is partly due to the protein denaturation and croos-linking reactions of myofibrillar proteins and also due to descication. In the case of fillet it is advised to pass the rigor-mortis before freezing in order to minimise the muscle contraction and toughness.

5. Protein denaturation

The most important change associated with freezing and frozen storage is denaturation of protein. The structural changes taking place in the protein molecule makes definite changes in the physical, chemical and biological properties of the protein. Curdling of protenaceous material observed during repaeted freezing and thawing is an important physical observation associated with protein denaturation. The loss in characteristic texture due to decrease in water holding capacity of protein brings about increased driploss in some species of fish. Dteriorative protein changes can be prevented by employing very low and constant temperature during storage and by inhibiting the oxidation of fat and descication of the product.

6. Fat oxidation during frozen storage

Fat oxidation is a serious concern during the frozen storage of fatty fishes. The unsaturated fatty acids of fish lipids are succeptible to oxidation and the end result is the formation of different kind of undesirbale compounds such as aldehydes, carbonyls and other secondary oxidation products. These products besides producing off flavours, reduce the nutritional value of the frozen stored products. Oxidised lipids interact with proteins reducing the nutritive value of the proteins considerably. Melonaldehyde is one of the major oxidationproducts and estimation of this compound by forming the thiobarbituric acid (TBA) complex is

the accepted method for monitoring the extent of lipid oxidation. In lipid oxidation, the first step leads to the formation of hydroperoxides, which are tasteless but can cause brown and yellow discolouration of the fish tissue. The degradation of hydroperoxides gives rise to formation of aldehydes and ketones. These compounds have a strong rancid flavour. Lipid oxidation is primarily non enzymatic in nature, recently the involvement of microsomal enzymes and lipoxygenase has been reported. This lipid oxidation takes place in fishes having more than 2% of the lipids eg. fatty fishes.

7. Cod store flavour

In some kind of frozen fish such as cod, prolonged cold storage lead to the development of unpleasant odour and flavour refered to as cold store flavour. The compound responsible for such flavour is identified as cis-4-heptenal. This compound is believed to be developed during the oxidation of phospholipids in the fish muscle. During storage, if the fish gets dried up, oxygen reaches the susceptible fattyacids readily and enhances the development of cold store flavour.

QUALITY DEFECTS IN CURED FISH PRODUCTS

Viji P.

Curing and drying are traditional low cost preservation techniques. The processing techniques varies with type, size, nature and condition of the fish. Improper handling practices can result in poor quality product due to spoilage and insect infestation. Though salt is known to have bactericidal activity, some bacteria are halophilic and can cause microbial spoilage. In addition to the action of halophilic bacteria, changes in protein, fat, attack of insect etc. causes quality problems in salt dried products . The common defect found in salt dried fish products is given below.

1. Case hardening:

During drying, if the temperature is high and the relative humidity is low, the outer surface of fish get overdried and the inner layers of fish will be still having water content. When case hardening takes place, the temperature of fish muscle increaes and result in cooking. This will result in a briitle final product. Case hardening can be prevented by proper maintenance of temperature and relative humidity during drying.

2. Rancidity and discoloration

Pigments, fat etc. in the fish are sucsceptible to oxidation. Prolonged drying of fish exposed to hot air can accelearte this process. So , air dried fish often faces discolaration and rancid odoures. Oxidation is more in oily fishes such as sardine, mackerel, anchovies etc. lipid oxidation in dried fish give rise to unfavourable odour and the color changes to brown. This is known as rust. Another type of discolartion is the non enzymatic browing developed through maillard reaction.

3. “Pink” or “red”

This is a common type of microbial spoilage in cured product which occures mostly during the warm season. The surface of the fish become covered with a red slime that gives off an unpleasant odour. This is brought about by the growth of halophilic bacteria such as *Halobacterium salinaria*, *Sarcina morrhuae* etc. The spoilage manifest first as a delicate pink sheen on the surface of fish. In the initial

stages, it can be rubbed or washed off easily without damaging the fish. However, in severly affected fish, it will reappear.

4. Mould attack

"Dun" is a type of mould development observed in salted fish when the relative humidity is above 75%. This is characterised by the appearance of colored spots, black, grey or brown, visible particularly on the fleshy side of the fish. This gives an appearance as if sprinkled with black pepper. This discoloration is unsightly and reduces the marketabilty of the product. Some times, the small spots develop a root type network into the interior of the fish flesh.

The mould, *Sporendonema epizoem* is the common causative agent for this type of spoilage. This mould can be relatively easily removed in the early stages. In cases of severe infection, dipping in 0.1 % sorbic acid will give some protection. Since solar salt is the source of contamination, effective control may be achieved by the use of good quality salt.

5. Insect infestation

Infestation by flies is a very common and serious problem faced by the salt fish trade. Infestation actually takes place during the early stages of drying. Adult female flies get attracted to the fish and lay their eggs on the fish surface. The emerging adults can reinfest more fish and the cycle continues. Infestation can be controlled to a greater extend by maintaining proper hygienic handling practices. This include keeping the handling and drying premises clean and fly proof, keep the fish completely covered during salting etc.

Fungal infestation in dried fish

6. Rust

Appearance of a color similar to that of rusted iron on the dried fish surface is a most commonly occuring phenominan in the tropical countries. Oily fishes such as sardine, mackerel etc. are particularly prone to rusting. Apart from the discoloration, rusted fish is characterised by an unpleasant racid odour and taste. This all changes are brought about by the oxidation of oil in the fish. The best method to prevent the occurance of rust in fish is to prevent it from contact with atmosphearic oxygen. Dried fish should be properly packed in oxygen impermeable packages.

7. Fragmentation

Cured and dried fish often become brittle and break during storage and transportation. This is refferred to as fragmentation. Denaturation of protein, hallowing the fish by insect attack, use of spoiled fish for drying etc. are the reasons for fragmentation. It can be reduced by using fresh fish and providing adequate packages during storage.

Characteristics	Requirements
Water activity at 25°C	:Less than 0.78
Salt content (% sodium chloride)	: Not less than 12%
Histamine content (Max)	:200 mg/kg
Acid insoluble ash (sand) on dry basis	:Not more than 1%

Quality requirements of dried fish

Suggested readings

Balachandran, K.K., 2001. *Post Harvest Technology of Fish and Fish Products*, Daya publishing house, New Delhi.

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BIOCHEMICAL QUALITY ASSESSMENT OF SEAFOOD

Viji P and **Jesmi Debbarma**

The quality changes during preservation and storage of fish and fishery products is of great significance, since the important attributes are influenced by the post harvest handling practices. During spoilage of fish, a number of chemical reactions take place in the fish muscle. In the chemical assessment of quality, various compounds formed are quantitatively determined and correlated with sensory characteristics. These compounds are produced in fish muscle by autolytic enzymes, putrefactive microorganisms or by chemical reactions such as lipid oxidation. During the course of spoilage, these compounds gradually get accumulated in the flesh and their quantification is an important means to measure the progress of spoilage. The appeal of biochemical and chemical methods for the evaluation of seafood quality is related to the ability to set quantitative standards. The establishment of tolerance levels of chemical spoilage indicators would eliminate the need to base decisions regarding product quality on personal opinions. The following is an overview of some of the most widely used chemical indices of seafood quality.

1. Total volatile base nitrogen (TVB-N)

TVBN measures the amount of volatile bases formed from solubilised nitrogen derivatives. It is a measure of decomposition of proteins. TVB-N in fish is mainly composed of ammonia and primary, secondary and tertiary amines. Bacterial catabolism of aminoacids in fish muscle results in the accumulation of ammonia and other volatile bases. Ammonia and primary amines are bound by formalin, therefore this fraction is called the formalin bound nitrogen (FBN). The trimethyl amine (TMA) represents the fraction, which is not bound by formalin. The TVB-N value is used as an index of quality for deciding the state of freshness of fish (along with TMA). A level of 35-40 mg 1VB-N /100g of fish muscle is usually regarded as the limit of acceptability, beyond which the fish can be regarded as spoiled. Generally, there is an increasing trend in TVBN values as the fish gets spoiled.

2. Trimethylamine (TMA)

Trimethylamine (TMA) is used to assess the freshness in marine fish. TMA is derived from trimethylamineoxide (TMAO) which is critical for osmo regulation in marine fish. TMAO is a tasteless non-protein nitrogen compound whose content varies with the season, size and age of fish. During spoilage, TMAO is reduced by enzymes to TMA. The concentration of amines in fish tissues is both time and temperature dependent and is related to the deterioration of fish. The determination of TMA as an indicator of freshness (actually of decay) has been a useful criterion for evaluating the quality of fish. TMA-N between 10-15 mg / 100g muscle is considered as the limit of acceptability for round, whole chilled fish. This index is not suitable for freshwater fish and heat treated fish products.

3. Biogenic amines as an index of spoilage

Biogenic amines have been proposed as markers to evaluate fish freshness. Fish muscle has the ability to support the bacterial formation of a wide variety of amine compounds which result from the direct decarboxylation of amino-acids. Most spoilage bacteria possessing decarboxylase activity do so in response to acidic pH, presumably so that the organisms may raise the pH of the growth medium through the production of amines. Biogenic amines are non-volatile compounds, which are found at very low level in fresh fish and their accumulation is related to bacterial spoilage which are thermally stable and can be used as indicator of poor quality of raw material in preserved products.

Histamine, putrescine, cadaverine and tyramine are produced from the decarboxylation of histidine, ornithine, lysine and tyrosine, respectively. Histamine has received most of the attention since it has been associated with incidents of scombroid poisoning in conjunction with the ingestion of tuna, mackerel, mahi-mahi. It is interesting tonote that most of the biogenic amines are stable to thermal processing, so their presence in finished canned products is a good indication that the raw material was spoiled prior to processing. Although the biogenic amines have been associated with fish spoilage legal limit has been established for histamine only. The European Union set a maximum average content of 100 mg/kg fish for canned products and trace for ripened products. The US Food and Drug administration lowered the limit from 100 to 50 mg/kg, recommending that not only histamine level but also other biogenic amine content had to be taken

into account. India has also limited maximum permissible level for histamine content in frozen fish 200 mg/kg. Less than 5 mg/kg is considered safe for consumption; 5 – 20 mg/kg is safe; 20-100 mg/kg is probably safe and > 100 mg/kg is toxic and unsafe for consumption. Incidence of histamine poisoning after eating fish are mainly due to poor quality of raw material.

4. Nucleotide Catabolites

After the death of fish, ATP is broken down over a period of days by enzymes present in the flesh, to different substances. The final stages of this process is the formation of a compound called hypoxamthine, which gradually increase with time and can be used as a measure of quality of fish. The nucleotide degradation products especially inosine monophosphate (IMP), hypoxantine (H_x) or K value clearly reflects the quality loss in fish. The presence of higher levels of IMP in the muscle indicates relatively high quality, whereas accumulation of inosine and hypoxathine is an indicator of poor quality. The amount of nucleotide degradation products is measured by the enzymic method or by High Pressure Liquid Chromatography (HPLC) method. K value is one of the most appropriate indicators of freshness. It is the percentage of the intact ATP present at death that has been converted by enzymic action into hypoxathine and its immediate precursor called inosine in the chain of decomposition of ATP. HPLC method is used to determine the K value. K value as an index of estimating the freshness of fish has become widely used in Japan.

K value can be defined as,

$$K = \frac{H_xR + H_x}{H_xR + H_x + ATP + ADP + AMP + IMP} \times 100$$

Where H_xR = Inosine

H_x = Hypoxathine

ATP = Adenosines triphosphate

ADP = Adenosines diphosphate

AMP = Adenosines monophosphate

IMP = Iosines monophosphate

5. Peroxide value

The highly unsaturated fatty acids found in fish lipids are very susceptible to oxidation. The primary oxidation products are the lipid hydroperoxides. These compounds can be detected by chemical methods, generally by making use of their oxidation potential to oxidize iodide to iodine or to oxidize iron(II) to iron(III). The concentration of the hydroperoxides may be determined by titrimetric or by spectrophotometric methods, giving the peroxide value (PV) as milliequivalents (mEq) peroxide per 1 kg of fat extracted from the fish. The most common method is based on iodometric titration which measures the iodine produced from potassium iodide (KI) by the peroxide present in fat. PV is a good guide to assess the quality of fat. Fresh oil should have PV 1 mg.oxygen/kg. On storage it may increase to 10 mg/kg.

6. Free fatty acids (FFA)

Fish muscle contains lipase, which is able to catalyse the hydrolysis of short chain triglycerides. Free fatty acids are suspected of deriving primarily from phospholipids, as the latter disappear with time of storage which can be affected by the action of bacteria, enzymes or non-enzymic catalysis. During spoilage, the amount of free fatty acids increases, which can be measured by reacting with alkali and is expressed as %oleic acid.

7. Thiobarbituric Acid Value (TBA Value)

TBA index is the most used indicator for advanced lipid oxidation. The peroxides formed may break down to carbonyls, form polymers, or react with protein, vitamins, pigments etc.. Lipid oxidation frequently contributes to flavour changes that occur during the storage of food and is one of the major degradative processes responsible for losses in quality of high-fat foods. The most widely used test for measuring extent of oxidative deterioration of lipids in muscle foods is the 2-thiobarbituric acid test or TBA test, which expresses lipid oxidation in mg of malonaldehyde/Kg of the sample . Malonaldehyde was shown to be a secondary oxidation product of polyunsaturated fatty acids. TBA measures the malonaldehyde produced during fat oxidation. TBA reacts specifically with malodadehyde to give a red chromogen which can be determined spectrophotometrically. The test can be performed in two ways either directly in

food followed by steam distillation and allowing the distillate to react with TBA reagent or by preparing an extract of the muscle followed by colour development. A reagent blank is also run simultaneously and is is measured at 538 nm. The TBA number is calculated as milligram of malonaldehyde per kg of sample, which is equal to 7.8 times of the optical density.

Suggested Books for reading:

Handbook of Natural Toxins. (1988) Anthony T.U. Marcel Dekker, INC.

Principle of total quality On Achonu, V.K. and Ross, J.E. (1994) St. Lucie Press, Florida.

Quality Assurance in Seafood processing (2002) T.S.G. Iyer, M.K. Kandoran, Mary Thomas and P.T. Mathew, Society of Fisheries Technologists (I), Cochin

Quality Assurance in the Fish Industry (1992), H.H. Huss *et al (eds)* Elsvier Science Publishers, BV

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METHODS FOR SENSORY EVALUATION OF FISH

Jesmi Debbarma

Sensory analysis is the most direct method for evaluating the freshness and therefore, the quality of fish. Sensory evaluation is defined as ' a scientific discipline used to evoke, measure, analyze and interpret reactions to characteristics of food as perceived through the senses of sight, smell, taste, touch and hearing' (Stone and Sidel, 1993). In sensory analysis, the scientific means of quantifying and interpreting the variations in the sensory characteristics of food such as appearance, odour, flavour and texture are evaluated through the human senses of sight, smell, taste, touch and hearing. Most sensory characteristics can only be measured meaningfully by humans. However, advances are being made in the development of instruments that can measure individual quality changes. With some practice, the pattern of changes in sensory characteristic between very fresh and very spoilt food can be easily and quickly by sensory means and the degree of freshness can be accurately determined.

Sensory analysis constitutes a reliable, rapid, reproducible and relative easy way to evaluate freshness. Moreover, it can be applied to all fish species being in most cases non-destructive.However, like any other methodology, it has to be applied in a systematically and standardized way to give reliable information that can be used as an evaluation method.Without appropriate sensory analysis, there is a high risk of market failure Sensory analysis is too frequently often overlooked as a requirement before product launch, and is often carried out to a poor standard.

Seven quality factors are the most important and reliable in the Organoleptic examination of fish factors,

1. General appearance
2. Appearance of flesh
3. Texture of raw fish
4. Odour of raw fish
5. Odour of cooked fish

6. Flavour of cooked fish

7. Texture of cooked fish

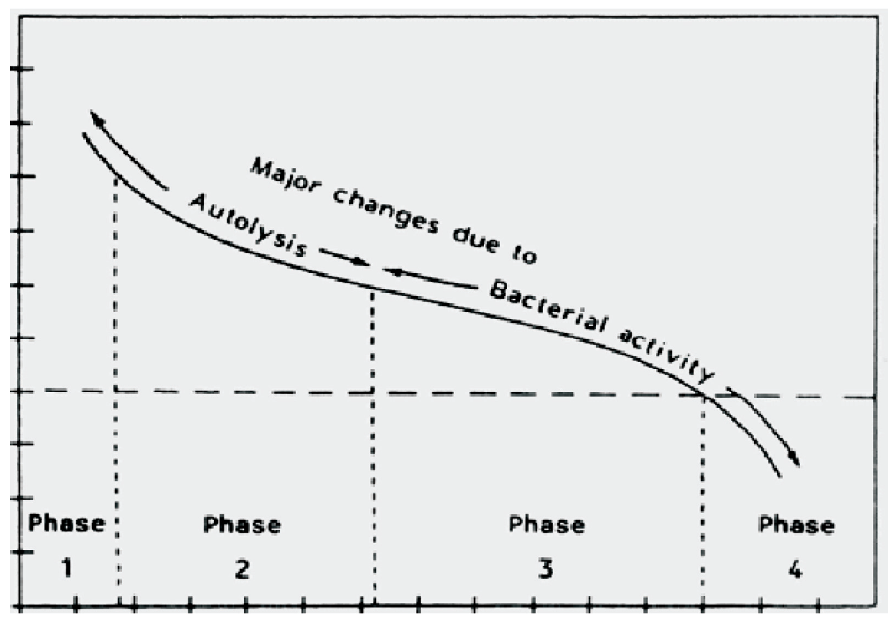
Application of sensory analysis includes quality control of raw materials and finished products, storage teats, development of new products, tand off flavour, aroma research, consumer test and hedonic test. There are two kinds of assessment generally followed,

a) Organoleptic testing (Subjective method) and

b) Sensory testing (Objective method)

A characteristic pattern of the deterioration of fish stored in ice can be found and divided into the following four phases:

- **Phase 1** The fish is very fresh and has a sweet, sea-weedy and delicate taste. The taste can be very slightly metallic. In cod, haddock, whiting and flounder, the sweet taste is maximized 2-3 days after catching.
- **Phase 2** There is a loss of the characteristic odour and taste. The flesh becomes neutral but has no off-flavours. The texture is still pleasant.



Scoring and grading for fish freshness

- **Phase 3** There is sign of spoilage and a range of volatile, unpleasant-smelling substances is produced depending on the fish species and type of spoilage (aerobic, anaerobic). One of the volatile compounds may be trimethylamine (TMA) derived from the bacterial reduction of trimethyl-aminoxide (TMAO). TMA has a very characteristic "fishy" smell. At the beginning of the phase the off-flavour may be slightly sour, fruity and slightly bitter, especially in fatty fish. During the later stages sickly sweet, cabbage-like, ammoniacal, sulphurous and rancid smells develop. The texture becomes either soft and watery or tough and dry.
- **Phase 4** The fish can be characterized as spoiled and putrid.

Quantification of sensory data requires the use of a scale, where the person can assess the deterioration that occurs continuously through varying degrees of intensity. Separate descriptive scales are used for each attribute and the range 10 to 0. The scale of ten is absolutely fresh and zero is completely putrid; anything below four is unacceptable.

Grading is the process of applying a categorical value to a lot or group of fish and fishery products.Grading has the advantage that it offers the possibility of selecting products for different qualities.Grading is simpler and less finely subdivided. I the EEC scheme of grading fish (chilled fish) four grades are given. In this, four grades, four grades (E,A, B and C) of freshness are laid down corresponding to the various stages of spoilage. E is the freshest and C is unfit for human consumptionThere are several grading methods used to assess freshness in fish and fish products such as:

1. The torry scoring system
2. The European Union schemes
3. The quality index method

The Torry Scoring System:

The first scoring method for use with fish and fishery products was developed at the Torry Research Station in the UK. The Torry scale is a 10-point scale originally developed to assess the eating qualities of cooked fish.Scores are given from 10 (for very fresh in taste and odour) to 3 (for spoiled fish) (Table 1). Scores below a 3 are considered unnecessary, as the fish is then not fit for human consumption.

The average score of 5.5 may be used as the limit for consumption.The Torry scale has been developed for lean, medium fat, and fatty fish species.

Table 1. Sensory score sheet for Cod (cooked) from gutted fish stored in melting ice

Score	Odour	Flavour	Texture, mouth feel and appearance	Score
10	initially weak odour of sweet, boiled milk, starchy, followed by strengthening of these odours	watery, metallic, starchy; initially no sweetness but meaty flavours with slight sweetness may develop	dry, crumbly with short tough fibres	**10**
9	shellfish, seaweed, boiled meat, raw green plant	sweet, meaty, creamy, green plant, characteristic		**9**
8	loss of odour, neutral odour	Sweet and characteristic flavours but reduced in intensity	succulent, fibrous; initially firm going softer with storage; appearance originally white and opaque going yellowish and waxy on storage.	**8**
7	wood shavings,	neutral		**7**
	woodsap, vanillin			
6	condensed milk, caramel, toffee-like	insipid		**6**
5	milk jug odours, boiled potato, boiled clothes-like	slight sourness, trace of 'off' flavours		**5**

4	lactic acid, sour milk, 'byre-like'	slight bitterness, sour, 'off' flavours	**4**
3	lower fatty acids (eg acetic or butyric acids), composted grass, soapy, turnipy, tallowy	strong bitter, rubber, slight sulphide	**3**

European Union Schemes:

In this scheme, three grades of freshness are established: E, A and B, corresponding to various stages of spoilage. E (Extra) is the highest possible quality; A is acceptable quality; while below B is the level where fish is considered unfit for human consumption (Table 2). This method gives rather limited information about the condition of the fish, as it is not species-related and does not take into account the differences between species. The EU-scheme is commonly accepted at auction levels however its use has been disputed.

Table 2. Criteria of EU schemes

	CRITERIA			
	Freshness Category			**Not Admitted**
	Extra	**A**	**B**	
Skin	Bright, iridescent pigment or opalescent, no discolouration	Pigmentation bright but not lustrous	Pigmentation in the process of becoming discoloured and dull	Dull pigmentation
Skin mucus	Aqueous, transparent	Slightly cloudy	Milky	Yellowish, grey, Opaque mucus
Gills	Bright colour, no mucus	Less coloured, transparent mucus	Brown/green becoming discoloured, thick opaque mucus	Yellowish, milky mucus

Peritoneum on gutted fish	Smooth, bright, difficult to detach from flesh	Slightly dull, can be detached from flesh	Speeckled comes away from flesh	Does not stick
Smell of gills and abdominal activity	Seaweedy smell	No smell of seaweed, neutral smell	Fermented, slightly sour	sour
Flesh	Firm and elastic, smooth surface	Less elastic	Slightly soft, less elastic	Soft, scales easily detached from skin, surface rather wrinkled.

Quality Index Method:

The QIM was developed at the Tasmanian Food Research Unit (TFRU) of the Commonwealth Scientific and Industrial Research Organization (CSIRO). QIM schemes are developed for individual species.Each attribute is scored from 0 to 3 by novice or experienced assessors with low scores indicating the best quality (Table 3). The sum of all attributes is called demerit points, or QIM index points. This value increases linearly with storage time in ice of a given fish, therefore, the linear relationship between the quality index (QI) and storage time on ice, makes it easy to calculate the remaining shelf-life of fish.

Table 3.Quality Index Method (QIM) schemes

Quality	Parameters	Description	Points
Whole fish	Skin colour/ appearance	Pearls-shiny, iridescent pigmentation	0
		Less pearl-shiny, yellowish, strips still distinct	1
	Odour	Neutral, pond, fresh fish, seaweed	0
		Melon, cucumber, green grass	1
		Cardboards, fishy, putid, rotten	2
	Texture	In rigor	0
		Firm, resilient, finger mark disappears immediately	1
		Soft, finger mark still persists after 3 seconds	2

Eyes	Pupil	Black, clear, bright, iridescent	0
		Dark gray, meat, dull	1
		Milky, cloudy, hazy, light, gray	2
	Shape	Convex, bulging	0
		Flat	1
		Concave, sunken	2
Gills	Mucus	Transparent, clear, none	0
		Milky, clotted	1
	Colour/ appearance	Bright red, red, burgundy	0
		Pale red, pink, light brown	1
		Brown, dull	2
	Odour	Pond, fresh fish, fresh rain	0
		Melon, cucumber, metallic	1
		Musty, fishy, putrid, rotten	2
Quality index (total score)			**0-14**

Suggested readings

1. Stone, H and Sidel, JL. 1993. Sensory Evaluation Practices. 2nd ed. Academic Press: San Diego.
2. Lawless, HT and Heymann, H. 1998. Sensory Evaluation of Food: Principles and Practices. New York: Chapman & Hall

BACTERIA OF PUBLIC HEALTH SIGNIFICANCE AND LABORATORY METHODS FOR THE DETECTION OF BACTERIA IN FISH / SHRIMP MEAT

Madhusudana Rao, B. and **Ahamed Basha, K.**

Food Safety

Food safety means that the food produced / processed is 'safe' for human consumption. Hazard is any biological, chemical or physical agent that may cause an unacceptable health risk to consumers if present in the product. Safe food means that the food is free from biological hazards (eg.harmful bacteria such as *Salmonella*, *Listeria monocytogenes*, *Vibrio cholerae,* viruses, worms, protozoa etc.), chemical hazards (harmful chemicals such as antibiotic residues, pesticides, heavy metals, dyes, cleaning compounds, food colours and additives other than permitted ones etc.)and physical hazards (foreign objects such as metal pieces, bolts, nuts, glass pieces, filth etc.).

Food safety regulations have been developed by all countries to ensure that the fish / shrimp that is available for human consumption is safe to eat. Non-compliance to standards leads to import refusals, detention, or destruction at the entry points of the importing country. Unsafe fish /shrimp products ontaining biological, chemical or physical hazards is not permitted to be sold in the markets.The food safety regulations for export of fish/ shrimp from India are governed by the Export Inspection Council of India [Order S.O. 729 (E), dated 21 Aug, 1995 subsequently amended vide orders 792(E), 722(E), 464(E), 1227(E) (EIC, 1995). However, if specific food safety standards are prescribed by the importing countries, then the standards of the importing countries must be invariably followed.

Fish and shrimp products that are meant for sale in India for human consumption are governed by the Food Safety and Standards Regulations (FSSR) of the Food Safety and Standards Authority of India (FSSAI), Government of India.

Table: Microbiological Requirements for Raw-Frozen Crustaceans (FSSAI, 2017)

	Sampling Plan		Limits (cfu/g)		Action in case of Unsatisfactory results
Hygiene Indicator Organisms	n	c	m	M	
Aerobic Plate Count (cfu /g)	5	3	$1x10^6$	$1x10^7$	Improvement in hygiene; Time-Temperature Control along value chain
Safety Indicator Organisms					
Escherichia coli	5	3	11 MPN/g	500 MPN/g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		

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

Bacteria are broadly categorized as Gram-positive (blue/purple colour) and Gram-negative (orange/red colour) based on their colour observed under microscope after being subjected to Gram staining. The important Gram-negative pathogens are *Salmonella, Shigella, Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus*, pathogenic *Escherichia coli* such as Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC), Enterohemorrhagic *Escherichia coli* (EHEC), Enteroinvasive *Escherichia coli* (EIEC) etc. The important Gram-positive pathogens

are *Staphylococcus aureus, Listeria monocytogenes, Clostridium botulinum, Bacillus cereus* etc.

Table: Bacteria of public health significance

Gram negative pathogens	Gram positive pathogens
• *Salmonella* • *Shigella* • *Vibrio cholerae s*erogroups O1 and O139 • *Vibrio parahaemolyticus* • *Vibrio vulnificus* • *Aeromonas hydrophila* • *Brucella* • *Yersinia enterocolitica* • *Campylobacter jejuni* • *Cronobacter* (*Enterobacter sakazakii*) spp. • *Coxiella burnetiid* • *Plesiomonas shigelloides* • *Francisella tularensis* • Pathogenic Escherichia coli Group • Enterotoxigenic *Escherichia coli* (ETEC) • Enteropathogenic *Escherichia coli* (EPEC) • Enterohemorrhagic *Escherichia coli* (EHEC) • Enteroinvasive *Escherichia coli* (EIEC)	• *Staphylococcus aureus* • *Listeria monocytogenes* • *Clostridium perfringens* • *Clostridium botulinum* • *Bacillus cereus* • *Streptococcus* • *Mycobacterium bovis* • *Enterococcus*

a) Salmonella: *Salmonella* is a motile, non-spore forming, Gram negative, rod-shaped bacterium. The genus *Salmonella* is divided into two species that can cause illness in humans viz., *S. enterica* and *S. bongori. Salmonella enterica*, which is of the greatest public health concern, is comprised of six subspecies: *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI). *Salmonella* is further subdivided into serotypes, based on the Kaufmann-

White typing scheme which differentiates *Salmonella* strains by their surface (O) and flagellar (H) antigenic properties. S. *enterica* subsp. *enterica* is divided into numerous serotypes. Salmonella causes typhoid disease which is characterized by high fever, diarrhoea, aches, headache, and lethargy (drowsiness or sluggishness) and up to 10% of people who don't get treatment may die. Typhoid fever is caused by serotypes *S.* Typhi and *S.* Paratyphi. Salmonella spreads through the fecal-oral route.

Sources of Salmonella: Intestinal tracts of vertebrates, including livestock, wildlife, domestic pets, and humans, and may also live in environments such as pond-water sediment, farm-irrigation water, soil and insects, factory equipment, hands, and kitchen surfaces and utensils. Cross contamination of fish / shrimp when a food handler does not adequately clean utensils, surfaces, equipment, and hands after they have come into contact with contaminated products. Cross contamination may occur at any point in the fish / shrimp processing unit.

b) ***Vibrio cholerae :*** *V. cholerae* is Gram negative, non-spore forming, comma shaped bacterium. It is the etiological agent of the dreaded disease 'cholera'. *V.cholerae* causes profuse watery diarrhoea, vomiting, and muscle cramps. The diarrhoea has a characteristic "rice water" appearance. The disease results in 30 to 40% mortality if rehydration therapy is not given. Virulence of *V. cholerae* serogroups O1 and O139 is due to the production of an enterotoxin called cholerae toxin (CT) and the toxin co-regulated pilus (TCP). Serogroups O1 and O139 were responsible for all the epidemic and pandemic cholera outbreaks. *V. cholerae* is excreted in great numbers in the faeces of cholera patients and convalescents. The disease is transmitted primarily by the faecal-oral route, indirectly through contaminated water supplies.

***Sources of V. cholerae*:** Cholera infections have been associated with wide variety of seafoods such as oysters, mussels, clams, crab, lobster, shrimp, squid and finfish. Raw, improperly cooked seafood, or cross contaminated by a raw product. Although cooking kills these bacteria, serogroups O1 and O139 can grow in

fish and shellfish that have been contaminated after cooking. In endemic areas infections occur through ingestion of water, ice, unwashed and contaminated food.

c) *Vibrio parahaemolyticus:* Gram-negative, curve-shaped rod frequently isolated from the estuarine and marine environments. It is a halophilic bacterium and requires at least 0.5% NaCl in all media, and 2% NaCl is optimal. *V. parahaemolyticus* is highly susceptible to low pH, freezing, and cooking. *V. parahaemolyticus* isolates from the environment are usually non-pathogenic. The pathogenic strains of *V. parahaemolyticus* are identified by the presence of one or both of the hemolysins TDH (thermostable direct hemolysin) and TRH (thermostable-related hemolysin). Diarrhea caused by *V. parahaemolyticus* is usually self-limiting. **Sources**: Consumption of raw or improperly cooked oysters, fin-fish, squid, octopus, lobster, shrimp, crab and clams.

d) *Vibrio vulnificus:* *V. vulnificus* is associated with various marine species, such as plankton, shellfish, crustaceans, and finfish. It is a halophilic bacterium that requires at least 0.5% NaCl in all growth media, and 2% NaCl is optimal. Death occurs in an average of 35% of septicemia cases (and 20% of wound infection cases). In healthy people, ingestion of this organism can cause gastroenteritis that generally remains localized and is self-limiting. Among susceptible people, the organism may cause primary septicemia (septic shock). Susceptible people include those with a predisposing condition; for example, those who are immunocompromised or have high serum iron levels (usually due to liver disease). *V. vulnificus* causes wound infections directly, either through wounds incurred while handling fish, crustaceans, or shellfish, or when a pre-existing wound is exposed to marine or estuarine waters harboring the organism. More than 90% of *V. vulnificus* illnesses in the U.S. are associated with consumption of raw Gulf Coast oysters. Ingestion of clams and shrimp also has been associated with disease.

e) *Listeria monocytogenes:* *L. monocytogenes* is aGram-positive, rod-shaped, facultative bacterium, motile and salt-tolerantbacterium.*L. monocytogenes* can survive below 1 p C and can grow at low temperatures (less than 3 p C) making it

an important organism in seafood industry. *L. monocytogenes* can be found in moist environment and is persistent in food-manufacturing environments. The genus *Listeria* contains 6 species: *L. monocytogenes*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, *L. ivanovii*,and *L. grayi* but only *L. monocytogenes* is commonly associated with human listeriosis. Listeriosis associated infection by *L. ivanovii*, and *L. seeligeri* is extremely rare in humans. *L. monocytogenes* bacterium causes two forms of disease. The first form manifests as non-invasive gastrointestinal illness and the infected persons suffer from nausea, fever, diarrhoea and is self-limiting. The second form manifests as a serious invasive illness that causes septicaemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion (2nd/3rd trimester) or stillbirth. **Sources**: Food workers, food contact surfaces, incoming air, raw materials, and food -processing environment.

f) ***Staphylococcus aureus:*** *S. aureus*is a Gram-positive, nonmotile, catalase-positive, small, spherical bacteria (cocci), which, on microscopic examination, appear in pairs, short chains, or bunched in grape-like clusters. *S. aureus* is a versatile human pathogen capable of causing staphylococcal food poisoning, toxic shock syndrome, pneumonia, postoperative wound infection, and nosocomial bacteremia. ***Sources***: Staphylococci are widely distributed in the environment. They can be found in the air, dust, sewage, water, milk, and food, or on food equipment, environmental surfaces, humans, and animals. Foods that require considerable handling during preparation and are kept slightly above proper refrigeration temperatures for an extended period after preparation are frequently involved in staphylococcal food poisoning. Unless heat processes are applied, staphylococci are expected to exist in any and all foods that are handled directly by humans or are of animal origin. Staphylococci are present in the nasal passages and throats and on the hair and skin of 50% or more of healthy individuals. Contamination may be introduced into foods by direct contact with workers with hand or arm lesions caused by *S. aureus*, or by coughing and sneezing, which is common during

respiratory infections. Food handlers are frequently the source of food contamination in staphylococcal outbreaks.

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ANTIMICROBIAL RESISTANCE (AMR) AND ITS DETECTION METHODS

Madhusudana Rao, B.* and **K. Ahamed Basha**

Antimicrobial resistance:

Antimicrobial resistance (AMR) is the non-responsiveness of microorganisms to antimicrobial agents in standard doses making it difficult to treat infectious diseases. Emergence of antibiotic resistance in bacterial pathogens is recognized as a major public health threat affecting humans worldwide and World Health Organisation has named antibiotic resistance as one of the three most important public health threats of the 21stcentury. AMR can affect anyone, of any age, in any country and is a threat to food security and sustainable development. Globally, AMR is responsible for 7,00,000 deaths annually and is predicted that AMR might cause 10 million deaths per year by 2050. Economy-wiseAMR might cause USD 100 trillion loss and 3.5% reduction in GDP by 2050.

A recent analysis of the effect of AMR in 204 countries and territories in 2019 had estimated that 4.95 million deaths were associated, and 1.27 million deaths were attributable to bacterial AMR. The main bacteria involved with the deaths were *Escherichia coli*, followed by *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*.

Antimicrobials are vital for the treatment of bacterial infections in humans, terrestrial animals and aquatic animals. Antibiotics exert their antibacterial action by several mechanisms such as inhibiting the bacterial cell wall synthesis (penicillins, cephalosporins, monobactams, carbapenems), disrupting the bacterial cell membrane (polymyxin, colistin, daptomycin), inhibiting protein synthesis (phenicols, macrolides, tetracyclines) or inhibiting nucleic acid synthesis or replication (sulfonamides, trimethoprim, quinolones, fluroquinolones).

More and more species of bacteria are becoming resistant to existing antibiotics. Multidrug resistant (MDR)bacteriumis a strain that hasacquired resistance to at least one agent in three or more antimicrobial classes. Extremely drug resistant (XDR)bacteria aresusceptible to only one or two antimicrobial classes and Pan drug resistant (PDR) bacteriaare non-susceptibile to all categories of antimicrobials. The MDR, XDR and PDR superbugs are emerging due to multiple mutations which have the brutal power to enhance morbidity and mortality. There is lack of interest by the pharmaceutical companies to develop new antibiotics as the bacteria were becoming rapidly resistant to newly introduced antibiotics, thus making the business less profitable. The mean duration of resistance was reported to be 6.9 years.

Antibiotic resistance in bacterium is classified as 'intrinsic resistance' and 'acquired resistance'. Intrinsic resistance refers to the natural existence of genes in the bacteria that make the bacteria resistant to that particular antibiotic. For example, resistance to penicillin is expressed by most Gram-negative bacteria. *Aeromonas* spp., commonly found in freshwater aquaculture environments, have been reported to have intrinsic resistance to ampicillin. In clinical settings, the resistance is usually 'acquired', wherein the bacterial population that was originally susceptible to an antibiotic acquires resistance. Acquired antibiotic resistance results, either from mutations in the chromosomal gene (vertical transmission) or due to acquisition of resistance genes from other bacteria in the environment (horizontal gene transfer, HGT). Horizontal gene transfer occurs through uptake of free DNA by a competent bacterial cell (transformation) or by mobilization of bacterial DNA from one bacterial cell to another by a bacteriophage (transduction) or by mobilization of DNA from a donor bacterium to a recipient bacterium through conjugative machinery (conjugation). HGT is the most relevant mode of resistance emergence and spread in bacterial populations.

Bacteria develop resistance to antibiotics by adopting different strategies such as modifying the antimicrobial molecule, preventing the antibiotic from reaching

the target site, changing the antibiotic target sites and bypassing the antibiotic target sites.

a) *Altering the antibiotic:*Bacteria produce enzymes such as acetyltransferase, phosphotransferase, adenyltransferase, that introduce chemical changes in the antibiotic molecule and modify them leading to loss of their antimicrobial property. This type of resistance was reported towards chloramphenicol, aminoglycosides and lincosamides

b) *Destroying the antibiotic:*Bacteria produce enzymes such as â-lactamases, Extended Spectrum â-Lactamases (ESBLs), that completely destroy the antibiotic making it incapable of executing its antibacterial function. This type of resistance was reported againstpenicillins, cephalosporins and monobactams,

c) *Target protection:* Bacteria alter the ribosomal conformation. This type of resistance was reported towardstetracycline, fluoroquinolones andfusidic acid.

d) *Target modification:*Bacteria modify the target site and decrease the affinity of the antibiotic for the target site. This type of resistance was reported against fluoroquinolone, rifampin and erythromycin

e) *Decrease permeability to prevent antibiotic entry:*Bacteria develop mechanisms to prevent the antibiotic from reaching its intracellular or periplasmic target. This mechanism limits the influx of substances from the external environment. This type of resistance was reported towards â-lactams, tetracyclines and some fluoroquinolones

f) *Efflux Pumps to flush out antibiotics from the bacterial cell:* Efflux pumps are transport proteins involved in the extrusion of toxic substrates. Efflux pumps have been characterized in both Gram-negative and Gram-positive bacteria. This type of resistance was reported against fluoroquinolones, â-lactams, carbapenems and polymyxins.

Detection of antimicrobial resistance

AMR is detected phenotypically by employing the disk diffusion assay (qualitative) or by determining the minimum inhibitory concentration (quantitative). Genotypic methods are used to detect the antibiotic resistance genes either by conventional PCR (qualitative) or real time PCR (quantitative).

Disk diffusion assay is performed as per Kirby-Bauer method by placing six discs of selected antibiotics on Mueller-Hinton agar plates seeded with the target bacterium (1.5 x 10^8cfu/ml). The inhibition zone size is measured and the results are interpreted as per internationally recognized guidelines such as Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial susceptibility testing (EUCAT).

The minimum inhibitory concentration (MIC) method determines the lowest concentration of an antibiotic that inhibits the growth of the target bacterial isolate.MIC test can be performed either by liquid broth dilution methods (tube dilution, microbroth dilution) or solid agar dilution methods. The preferred method is the microbroth dilution method in 96 well plates using cation adjusted Mueller-Hinton broth and using the target bacterium at an inoculum level of 5 x 10^5cfu/ml.

E-test (epsilometer test) is also a quantitative method that uses an inert strip with a pre-defined gradient of specific antibiotic that is placed on a Mueller-Hinton agar plate seeded with the target bacteria. The MIC is derived from the symmetrical inhibition ellipse that is seen after the incubation period. It is necessary to regularly employ quality control strains to evaluate the performance of the antibiotic susceptibility test method in the laboratory as several factors such as media composition, inoculum size, quality of antibiotic discs, incubation temperature etc., severely affect the test results.The commonly used quality control strains were *E.coli* ATCC25922, *Pseudomonas aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923 and *Streptococcus pneumoniae* ATCC49619.

Interpretative criteria can be of two types viz., clinical breakpoints or epidemiological cut-off values. Clinical breakpoints categorize a bacterial isolate as

either sensitive (S), intermediate (I) or resistant (R) and indicate the most probable outcome of specific therapy of a specified infected host. 'Sensitive' indicates that using that particular antibiotic would be helpful in treating the infection whereas 'resistant' suggests that the clinical outcome of using that specific antibiotic would not have a positive therapeutic outcome.

On the other hand, epidemiological cut-off values categorize bacterial isolates as wild type (fully susceptible) or non-wild type (reduced susceptibility) when compared to other members of its species. Advanced tools such as antibiotic resistance gene detection chips,microarray expression analysis, transcriptomics, metatranscriptomics, functional metagenomics, next-generation sequencing, single moleculereal-time sequencing methods werealso used for diagnostics and AMR deciphering.

The 'One Health' strategy i.e. involving the human, animal, food and the environment was adopted by the 'Quadripartite' namely Food and Agriculture Organization (FAO), World Health Organization (WHO), World Organisation of Animal Health (WOAH) and United Nation Environment Programme (UNEP) for addressing the menace of AMR. The Global Action Plan on AMR in 2015 and India's National Action Plan on AMR in 2017 were prepared with the 'One Health' centric approach. On similar line Kerala (2018), Madhya Pradesh (2019), Delhi (2020) and Andhra Pradesh (2022) have prepared the State Action Plans to combat AMR. This indicate the significance and thrust being given at the global, national and state level to mitigate AMR and save human and animal life.

HAZARD ANALYSIS CRITICAL CONTROL POINT (HACCP) SYSTEM: AN OVERVIEW

Madhusudana Rao, B.*, Jesmi Debbarma and **K. Ahamed Basha**

HACCP is an acronym that stands for Hazard Analysis Critical Control Point. HACCP is a systematic approach to the identification, evaluation, and control of food safety hazards. It is aproactive strategy where hazards are identified and assessed, and control measures aredeveloped to prevent, reduce, or eliminate a hazard. The goal of HACCP is to produce foodproducts that are 'SAFE' for human consumption. The result of the implementation of theHACCP Plan is known as the HACCP System. Use of HACCP system will move a foodproducing company from sole retrospective end product testing approach towards a preventivequality assurance approach.

HACCP Definitions

- **HACCP:** A systematic approach to the identification, evaluation, and control of food safety hazards.
- **Prerequisite Programs:** Procedures, including Good Manufacturing Practices, thataddress operational conditions providing the foundation for the HACCP system.
- **Risk:** an estimate of the likelihood of the occurrence of a hazard
- **Severity of risk:** the seriousness of a hazard if not properly controlled
- **Hazard:** any biological, chemical or physical property that may be expected to cause anunacceptable health risk to consumers if present in the product
- **Hazard Analysis:** The process of collecting and evaluating information on hazardsassociated with the food under consideration to decide which are significant and mustbe addressed in the HACCP plan.
- **HACCP Team:** The group of people who are responsible for developing, implementingand maintaining the HACCP system.

- **Critical control point (CCP)** – a specific point in a process where control can be appliedto eliminate or reduce the risk of a hazard to an acceptable level
- **CCP Decision Tree:** A sequence of questions to assist in determining whether acontrol point is a CCP.
- **Control Measure:** Any action or activity that can be used to prevent, eliminate orreduce a significant hazard.
- **Monitor:** To conduct a planned sequence of observations or measurements to assesswhether a CCP is under control and to produce an accurate record for future use inverification.
- **Validation:** That element of verification focused on collecting and evaluatingscientific and technical information to determine if the HACCP plan, when properlyimplemented, will effectively control the hazards.
- **Verification:** Those activities, other than monitoring, that determine the validity of theHACCP plan and that the system is operating according to the plan.
- **HACCP Plan:** The written document which is based upon the principles of HACCP andwhich delineates the procedures to be followed.

History of HACCP:

The concept of HACCP was first used in the US space program to ensure safe food forastronauts without relying on end-product testing. It was developed in 1960s by a team fromPillsbury company to produce 'zero defects' food products for the NASA astronauts. HACCPwas adopted in 1973 by the USFDA for low acid canned food regulations (pH < 4.6). TheHACCP standard developed by the U.S. National Advisory Committee on the MicrobiologicalCriteria for Food (NACMCF) was later adopted by the Codex Alimentarius Commission andpublished as the first international HACCP standard in 1992. In 1997, the USFDA madeHACCP mandatory for all seafood processors in the US as well as for those foreign plantsexporting to

the US. The Canadian Food Inspection Agency (CFIA) made HACCP mandatoryfor all Canadian seafood processors in 1998. Similar legislation has been imposed in the EUfor food processors within the EU and those exporting to EU countries. The CodexAlimentarius Commission – an organization linked to the WHO (World Health Organization)and FAO (Food and Agriculture Organization of the United Nations), with representatives frommore than180 countries – has published the HACCP approach for managing food safety risks.This approach is being adopted by government agencies and industry organizations globally.Although specific regulations in different parts of the world continue to evolve, they typically feature some common elements from HACCP:

Seven fundamental principles of HACCP

Principle 1 - Conduct hazard analysis for each product.

Principle 2 - Identify Critical Control Points

Principle 3 - Establish critical limits

Principle 4 - Establish CCP monitoring procedures

Principle 5 - Establish Corrective action when critical limits have been exceeded

Principle 6 - Establish HACCP verification procedures

Principle 7 - Establish effective record keeping and documentation

Twelve Steps to Implement HACCP

1. Assemble HACCP team
2. Describe product
3. Identify intended use
4. Construct process Flow Diagram and Plant Schematic
5. On-site verification of Flow Diagram and Plant Schematic
6. List hazards associated with each step – (HACCP principle 1)

7. Apply HACCP decision tree to determine CCP – (HACCP principle 2)

8. Establish critical limits – (HACCP principle 3)

9. Establish monitoring procedures - (HACCP principle 4)

10. Establish deviation procedures - (HACCP principle 5)

11. Establish verification procedures - (HACCP principle 6)

12. Establish record keeping/documentation for steps 6-11 - (HACCP principle 7)

Step 1 - Assemble HACCP Team: A multi-disciplinary HACCP team comprising ofindividuals with different specialties is integral for the successful implantation of HACCP inany seafood processing unit. The members of the team include people from maintenance,quality control, production, cleaning and sanitation, people involved in the day- to-day plantoperations. The HACCP team has to be led by a team leader who must be well-trained, have areasonable scientific background and have the ability to motivate and work well with others.The team must have access to reliable technical information. The main duties of the HACCPteam are to develop the HACCP plan, verify the HACCP plan, implement and continuallyrevise the HACCP plan to accommodate changes. The team approach is preferred as a singleperson cannot be an expert on all operations of the processing unit. Moreover, team approachencourages 'ownership' of the decisions.

Steps 2 & 3- Description and Intended Use of Product: The fish products that are to bemanufactured have to be described in details with its distribution chain. This providesinformation on the ingredients, processing methods, and distribution methods (frozen,refrigerated, or at ambient temperature). Identify how the fish are stored after receipt, how thefinished product will be shipped, how the finished product will be packaged, how the productsare intended to be used and finally identify the intended consumer. The consumers of the foodalso have to be described thoroughly. These may be the general public or a segment of thepopulation such as infants or the elderly.

Step 4-Construct a process flow diagram: A flow diagram describes the process for eachproduct. This diagram provides a clear, simple outline of the steps involved in the making theproduct. A block flow diagram is usually sufficient. The flow diagram details all the processactivities including inspections, transportation, storage and delays in the process as they "flow"from receipt to distribution.

Step 5- On site verification of the process flow diagram: The HACCP team should performan on-site review of the operation to verify the accuracy and completeness of the flow diagram.Perform a walk-through of the process to make sure all process steps are covered. It should bedone by all members of the HACCP team during all stages and hours of operation

Step 6-Hazard Analysis (HACCP Principle 1): The HACCP team reviews the ingredientsused, activities at each processing step, and then makes a list of food safety hazards that arereasonably likely to cause injury or illness if not controlled. Hazard is any biological, chemicalor physical agent that may cause an unacceptable health risk to consumers if present in theproduct. Examples of biological hazards are pathogenic bacteria, viruses, parasites; examplesof chemical hazards are heavy metals, pesticides, antibiotic residues, dyes; examples ofphysical hazards are metal pieces, glass pieces. The hazards may be species related or processrelated. These food safety hazards may already be present in the raw material or, on the otherhand, these may be introduced during the subsequent processing stages, which thus adverselyaffect the hygiene and safety status of the product. The HACCP team must identify steps in theprocess where significant hazards may occur, identify the type and nature of contamination,the stage at which it might occurred (either during or after processing). The team must estimateboth risk and severity of hazards. The risk assessment is based upon experience, epidemiological data and technical information.

Five steps are involved in a hazard analysis:

i. List process steps

ii. Identify potential food safety hazards

iii. Determine if the hazard is significant

iv. Justify the decision

v. Identify control measure

The HACCP team should identify the preventive measures for each hazard to reduceprobability of risk. Biological hazards are controlled through time/ temperature controls,cooking, freezing, fermentation and/or pH controls, adding salt or other preservatives, dryingor other processing techniques. Chemical hazards (natural toxins, pesticides, drug residues,unapproved food and colour additives, histamine) are mainly controlled through source controls,time/temperature controls, production controls and labelling controls. Physical hazards (metal,glass) are controlled through source controls and production controls.

Step 7 - Identify the Critical Control Points (CCP's) (HACCP Principle 2): A CCP is apoint, step or procedure which can be applied to prevent, eliminate or reduce hazards to anacceptable level. Every significant hazard must have a corresponding CCP and a "decisiontree" is used to determine if a process point is a CCP or not.

The decision tree follows a series of questions.

Q1: Does this step involve a hazard of sufficient risk and severity to warrant its control?

Yes – go to Q2

No – Not a CCP

Q2: Does a control measure for the hazard exist at this step?

Yes – go to Q3

No – Is control of step necessary for safety?

Yes – Modify the step process or product

No – Not a CCP – Stop*

Q3: Is control at this step necessary to prevent, eliminate or reduce the risk of the hazard to consumers?

Yes – CCP

No – Not a CCP – Stop*

The examples of CCPs in a fish processing unit

- Reception of raw materials
- Cooking:
- Metal detection point
- Labelling

CCPs are Product and Process Specific. CCPs may change with differences in plant layout,product formulation, process flow, change in equipment, ingredient selection and sanitationand support programs.

Step 8- Establish Critical Limits (HACCP Principle 3): Critical limit is a maximum and/orminimum value to which a biological, chemical or physical parameter must be controlled at aCCP to prevent, eliminate or reduce to an acceptable level the occurrence of a food-safetyhazard. Critical limits are boundaries which cannot be exceeded if the hazard is to be prevented,eliminated or minimized. Critical limits are scientifically determined based on informationavailable in journal articles, food science texts, microbiology texts, regulations and guidelines.Examples: Cooking temperature of 99°C for a specified time depending on the product size toattain core temperature of 72°C to control *Listeria monocytogenes*.

Step 9- CCP Monitoring (HACCP Principle 4): Monitoring involves a planned sequence ofobservations or measurements to assess whether a CCP is under control. These observationsare used to determine whether or not corrective action is required. Monitoring facilitatestracking of the operation, indicates when there is a loss of control and a deviation occurs andprovides written documentation for use during the verification process. Examples of CCPmonitoring are time and

temperature of cooking process, water activity (aw), pH, internalproduct temperature, salt concentration in brine, metal inclusion screening.

Step 10- Corrective Actions (HACCP Principle 5): Corrective actions are the plannedactions that are to be undertaken when monitoring indicates that there is a deviation from anestablished critical limit. A failure to meet a required critical limit for a critical control point isknown a deviation. There is a need for clear corrective action protocols and chain of commandmust be emphasized. The corrective actions taken must bring the CCP back under control toensure that the production process will not cause consumer illness. All the corrective actionstaken to fix the problem that caused the deviation and restore process control must bedocumented and these records will help the firm identify recurring problems. The aim ofcorrective actions is to re-establish control of the process so that production can start again assoon as possible without further deviations. However, it needs to be emphasized that whencritical limit deviations frequently reoccur then, the process and the HACCP plan must be reevaluated.Examples of corrective actions include adding more salt to the brine solution; recookingif possible if the internal temperature was not achieved;

Step 11 - Verification (HACCP Principle 6): Verification includes those activities, other thanmonitoring, that determine the validity of the HACCP plan and that verify the system isoperating according to the plan. The purpose of verification is to provide a level of confidencethat the plan is based on solid scientific principles and control the hazards associated with theproduct and process, and is being followed. The type of verifications are Validation, CCPverification activities, HACCP system verification and verification by the regulatory agencies.Validation involves establishing the scientific basis for the HACCP plan. Validation isperformed initially and whenever there are changes in raw materials, changes in product orprocess, recurring deviations, new information on hazards or control measures, newdistribution or consumer handling practices. A validation example might be the documentation used to select a cook step to control salmonella in a ready-to-eat product, the minimum time and temperature needed to cook the product,

and the frequency of temperature monitoring to ensure safety. The second part of verification is to "verify" that the validated plan is being followed correctly. Examples of "verifying" include: calibration of process monitoring instruments, direct observation of monitoring activities and corrective actions, and review of records generated and maintained in accordance HACCP Plan. HACCP system is verified annually or whenever there is an occurrence of a system failure or significant change in product or process.

Step 12 - Record Keeping (HACCP Principle 7): Systematic record keeping system with filing system is a vital component of the HACCP system. Records pertaining to HACCP plan and supporting documentation, records of CCP monitoring, records of corrective actions, records of verification activities, sanitation Control records, importer verification records are maintained in fish processing units.

Conclusion

The future of fisheries depends on the production of safe and wholesome products, and this goal can be achieved by the strict enforcement of HACCPbased practices, during primary production /harvest stage, processing stage in fish processing units, distribution to domestic and international markets and storage during retail sale. The aim of quality assurance is to ensure that a product conforms as closely as possible and consistently to that standard at all times.

QUALITY AND SAFETY STANDARDS/ REQUIREMENTS FOR FISH AND FISH PRODUCTS

Madhusudana Rao, B.*, Viji P.,

Nearly 90% of the total fish production of India is marketed within the country. Several food standards have been laid down by the Food Safety Standards Authority of India (FSSAI) to ensure the quality of fish and fish products marketed throughout India. These standards have been introduced to protect the consumer's health and are necessary to achieve minimum standards of hygiene and cleanliness in fish handling, processing and marketing. Following are the quality requirements given by FSSAI for different kind of fish and fish products.

Table 1. Microbiological parameters for Chilled Finfish

	Sampling Plan		Limits		Action in case of unsatisfactory results
Hygiene Indicator Organisms					Improvement in hygiene; Time-Temperature Control along value chain
	n	c	m	M	
Aerobic Plate Count	5	3	$5x10^5$cfu /g	$1x10^7$ cfu/g	
Safety Indicator Organisms					
Escherichia coli	5	3	11 MPN/g	500 MPN/g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		

Table 2. Microbiological parameters for Chilled shrimp (Crustaceans)

	Sampling Plan		Limits		Action in case of unsatisfactory results
	n	c	m	M	Improvement in hygiene; Time-Temperature Controlalong value chain
Hygiene Indicator Organisms					
Aerobic Plate Count	5	3	1x106cfu/g	1x107cfu/g	

Safety Indicator Organisms					
Escherichia coli (MPN)	5	3	11 /g	500 /g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		

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

Table 3. Microbiological Requirements for Raw-Frozen Crustaceans (FSSAI, 2017)

	Sampling Plan		**Limits (cfu/g)**		**Action in case of Unsatisfactory results**
Hygiene Indicator Organisms	n	c	m	M	
Aerobic Plate Count (cfu /g)	5	3	$1x10^6$	$1x10^7$	Improvement in hygiene; Time-Temperature Control along value chain
Safety Indicator Organisms					
Escherichia coli	5	3	11 MPN/g	500 MPN/g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		

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

Table 4. Microbiological Requirements for Cooked-Frozen Crustaceans (FSSAI, 2017)

	Sampling Plan		Limits (cfu/g)		Action in case of Unsatisfactory results
Hygiene Indicator Organisms	n	c	m	M	
Aerobic Plate Count (cfu/g)	5	2	$1x10^5$	$1x10^6$	Improvement in hygiene; Time-Temperature Control along value chain
Coagulase positive Staphylococci (cfu/g)	5	2	$1x10^2$	$1x10^3$	
Safety Indicator Organisms					
Escherichia coli	5	2	1 MPN/g	10 MPN/g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		
Listeria monocytogenes	5	0	Absent/25g		

Table 5. Microbiological Requirements for Battered and Breaded shrimp products (FSSAI, 2017)

	Sampling Plan		Limits (cfu/g)		Action in case of Unsatisfactory results
Hygiene Indicator Organisms	n	c	m	M	
Aerobic Plate Count (cfu/g)	5	2	$1x10^5$	$1x10^7$	Improvement in hygiene; Time-Temperature Control along value chain
Coagulase positive Staphylococci (cfu/g)	5	1	$1x10^2$	$1x10^3$	
Yeast and Mold count	5	0	100		
Safety Indicator Organisms					
Escherichia coli	5	2	11 MPN/g	500 MPN/g	
Salmonella	5	0	Absent/25g		
Vibrio cholerae (01 and 0139)	5	0	Absent/25g		
Listeria monocytogenes	5	0	Absent/25g		

Table 6. Antibiotic residue in farmed shrimp / crustaceans (FSSAI, 2011)

Chemical hazardAntibiotics	Maximum Residue limit (MRL)
Tetracycline	0.1 ppm
Oxytetracycline	0.1 ppm
Trimethoprim	0.05 ppm
Oxalinic Acid	0.3 ppm
Chloramphenicol	Below MRPL*
Nitrofurans (metabolites)	Below MRPL

Table 7. Heavy metals in fin fishes and crustaceans

Lead		Cadmium	
1. Fin fishes	: 0.3 ppm	1. Fin fishes	: 0.3 ppm
2. Crustaceans	: 0.5 ppm	2. Crustaceans	: 0.5 ppm
3. Cephalopods	: 1 ppm	3. Cephalopods	: 2 ppm
4. Bivalve mollusks	: 1.5 ppm	4. Bivalve mollusks	: 2 ppm
5. Canned fish	: 5.0 ppm		
Mercury		**Arsenic**	
1. Non predatory fishes	: 0.5 ppm	1. Fin fishes	: 76 ppm
2. Predatory fishes	: 1.0 ppm	2. Crustaceans	: 76 ppm
3. Crustaceans	: 0.5 ppm	3. Molluscs	: 86 ppm
4. Cephalopods	: 0.5 ppm		
5. Bivalve mollusks	: 0.5 ppm		
Chromium		**Tin·**	
All fishery products:	12 ppm	Canned fish	: 200 ppm

Table 8. Microbiological Requirements for Dried Fishery Products (FSSAI, 2017)

	Sampling Plan		Limits (cfu/g)		Action in case of Unsatisfactory results
Hygiene Indicator Organisms	n	c	m	M	Improvement in hygiene; Selection of raw material; Adequate drying (water activity ≤ 0.78)
Aerobic Plate Count	5	0	$1x10^5$	-	
Yeast & mold count	5	2	100	500	
Safety Indicator Organisms					
Escherichia coli	5	0	20 MPN/g		
Salmonella	5	0	Absent/25g		

Table 9. Requirements for Edible fish powder (FSSAI, 2017)

Sl. No	Characteristics	Requirement
1	Moisture, Percentage by weight, Maximum	10
2	Crude protein content (Nx 6.25) percentage by weight on dry basis, minimum	65
3	Total available lysine (g/100 g of protein), minimum	6
4	Fat content on dry basis (percentage by weight), maximum	6
5	Ash on dry basis percentage by weight , Max.	18
6	Acid insoluble ash on dry basis, % by weight ,Maximum	0.5

Table 10. Requirements for Fish Pickles (FSSAI, 2017)

Sl. No	Characteristics	Requirement
1	Fluid portion % by weight, maximum	40
2	pH	4.0-4.5
3	Acidity as acetic acid of fluid portion, % by weight maximum	2.5-3.0
4	Sodium chloride, % by weight, maximum	12.0

ANNEXURE I: PROTOCOLS

A.Biochemical quality assessment

1.Total Volatile Base Nitrogen (TVBN)

As fish undergoes spoilage, the content of TVBN increases. TVBN is used to determine early stages of spoilage.

Principle:-

Volatile basic nitrogen content is mainly constituted by Ammonia. When TCA extract of the sample is treated with saturated Sodium carbonate, Ammonia will liberated which is then trapped in N/100 H_2SO_4 in the Conway dish. The excess acid in inner chamber is back titrated with N/100 NaOH. The calculated value gives the TVBN of the sample.

Reagents:-

1. N/100 H_2SO_4 & N/100
2. Saturated Sodium carbonate solution.
3. Mixed indicator.

Procedure:-

Preparation of TCA extract:-

Weigh 10 g of fresh muscle sample into a mortar. Add 10 ml. of 20% TCA and ground well. Filter, using Whatman's filter paper No.1 in 50 ml. standard flask. Repeat the extraction with 1% TCA & filter. Collect the washings and make the volume 50ml.

Analysis

1. TVBN is estimated by the micro diffusion Conway method. In the inner chamber of the conway unit place 1 ml. of N/100 H_2SO_4 and in the outer chamber, 1ml of TCA extract of the sample.

2. Cover the Conway dish with the glass cover smeared with petroleum jelly to give air-tight contact along the outer contact-ring of the unit.
3. Keep just open to draw 1ml of saturated Na2CO3 in the outer chamber of the unit, then closed the glass plate to air tight.
4. Mix gently by lateral circular movement.
5. Allow the unit undisturbed to stand overnight (or at 37^{0}C incubation for 2 hrs.).
6. The acid in the inner chamber is titrated against N/100 NaOH using two drops of mixed indicator, the indicator changing from red to green at the end point- (B).
7. Titrate a reagent blank also by taking standard acid at the central compartment- (A)
8. Perform the assay in duplicate for each sample.

Calculation:-

Value(A-B) is the vol. of N/100 acid used up by volatile base.

(1ml of N/100 acid = 0.14 mg of Nitrogen).

$$\text{TVBN mg \%} = \frac{(A-B) \times 0.14 \times 50 \times 100}{\text{Wt. of sample} \times 1}$$

2.Tri-methyl Amine (TMA)

Principle:-

Tri-methyl amine is a non-protein nitrogenous volatile compound.The quantity of TMA formed is depends primarily upon the concentration of its precursor, TMA-O in the fish muscle. TMA-O is reduced during spoilage to TMA. The TMA is often determined by the Conway micro-diffusion technique.

Procedure:-

The same procedure is adopted as TVBN, except that 0.5 ml Neutralized Formalin (prepared by shaking formaldehyde with Magnessium carbonate and filtering through Whatman 40 no. filter paper) is added to the outer chamber and swirled to mix before adding Sat. Sod. Carbonate. Formaldehyde is added to fix all the bases except TMA.

Calculation:-

TMA is also expressed in mg %.

$$\text{TMA mg \%} = \frac{(A - B) \times 0.14 \times 50 \times 100}{\text{Wt. Of sample} \times 1}$$

3.FREE FATTY ACIDS (FFA)

The deterioration of lipids has always been of primary concern to fishery technologists. Degradation of lipids falls into two categories: oxidation which leads to of odours and flavours and hydrolysis which splits off free fatty acids. FFA gives a measure of hydrolytic rancidity.

Principle:-

Fat spoilage can be assessed by estimating the free fatty acids (FFA) and peroxide value (PV) on a common chloroform extract. The FFA in the sample extract is diluted with alcohol and neutralized by titration with sodium hydroxide. The FFA are expressed as % Oleic acid on the extracted fat.

Reagents:-

1. Chloroform.
2. Anhyd. Sod. Sulphate
3. Neutral Ethyl alcohol (Neutralised with NaOH)
4. Phenolphathalein indicator.
5. 0.01N NaOH

Procedure:-

1. Take about 10 g of fresh muscle sample in a mortar & grind well with unhydrous Na_2SO_4 untill all water is removed.
2. Transfer this into 250 ml. Iodine flask. Add to this 100 ml. Chloroform & keep 30min. in dark.
3. Filter the chloroform extract using filter paper and make the vol. 100ml with chloroform.
4. Weigh 2 nos. of 50 ml. conical flasks. Add 20 ml. of chloroform extract in each conical flask.
5. Evaporate the extract in water bath & then dry them for 3 h in Hot air oven at 100^0C.
6. Cool and weigh the conical flask. This will give the fat content (M) in 20 ml of chloroform extract.
7. Add 10 ml. of warm, neutral alcohol & dissolve the fat.
8. Add 1 drop of phenolphthalein indicator & titrate against 0.01 N NaOH.

Calculation:-

FFA (as oleic acid on extracted fat), % (m/m)

$$FFA\ \% = \frac{V \times N \times 28.2}{M} \times \frac{100}{20} \times \frac{1}{\text{Wt. of sample}}$$

Where: M = Fat content in 20 ml.of chloroform extract

V = Vol. in ml. of NaOH

N = Normality of NaOH

28.2 = milliequivalent weight of oleic acid (include factor of 100 for %).

4. Peroxide Value (PV)

Principle:-

During oxidation of fat peroxide is formed. Peroxide value gives measure of oxidative rancidity. The peroxide value is a measure of peroxides contained in the oil.The peroxide value is usually determined volumetrically by method which depends on the reaction of potassium iodide in acid solution with the peroxide oxygen followed by titration of the liberated iodine with Sodium thiosulphate solution.

Reagents:-

1. Glacial acetic acid

2. 1 % starch solution

3. N/100 Sodium Thiosulphate solution

4. Potassium Iodide

Procedure:

1. In a 250 ml. Iodine flask, take 20 ml. of chloroform extract (prepared in FFA)
2. Add about 30 ml of glacial acetic acid and 1 g of KI &keep in dark for about 30 min. with occasionally swirling.
3. Take out and add 1 cc. 1% starch solution.
4. Titrate liberated iodine with N/100 Sod. Thiosulphate solution.

Calculation:-

$$\textbf{PV}\ \% = \frac{V \times N}{M} \times \frac{100}{20} \times \frac{100}{\text{Wt. of sample}}$$

V = ml of Sod. Thiosulphate solution used.

N = Normality of Sod. Thiosulphate.

M = Fat content in 20 ml chloroform extract.

5. Thiobarbituric Acid (TBA)

Principle:-

Oxidised lipids are formed as fats become rancid. Thiobarbituric acid will react with these fatty lipids to form a red – colored complex which can be determined spectrophotometrically. Malonaldehyde is one of the end products of oxidative rancidity and is believed to be involved in the reaction with TBA. Therefore the TBA value is expressed as mg malonaldehyde per Kg sample. The TBA test is applicable to fatty foods (e.g. meat) as well as fats and oils.

Reagents:-

- TBA reagent:- 0.2883gm in 100ml of 90% glacial acetic acid.

Procedure:-

1. Weigh 10 g of preapared sample in a round bottom flask . Intothis add a glass bead and 100ml solution (3ml 2:1 HCl + 97 ml DW = 100ml) & mix.
2. Collect 50 ml distillate by steam distillation within 10 min.
3. Pipette 5ml of distillate into a glass stoppered tube, add 5mlTBA reagent, stopper, shake and heat in boiling waterbath for 40min.
4. Preapare a blank similarly using 5ml DW with 5ml reagent.
5. Then cool the tubes in water for 10min. and measure the absorbance (A) against the blank at 538nm.

Calculation:-

TBA no. (as mg malonaldehyde / Kg sample) = 7.8 x A x 50

Wt. of sample 5

= 7.8 x A

7.8 is the TBA standard factor.

6. Free Alpha – amino Acids

In Crustaceans, the free alpha – amino acid is upto 40% of the NPN and in teleosts is only 6%. The attractive flavour invariably present in prawns and other crustaceans is attributable to their comparatively higher contents of free amino acids. The comparatively quicker rates of spoilage occurring in invertebrates than in teleosts may be attributed to the presence of large quantities of free amino – acids in their muscles.

Principle :-

The method depens on the formation of soluble copper compounds through the complex reaction between the amino acids and excess copper in the form of $CuSO_4$. The amount of copper taken into solution by amino acids or similar material is determined iodometrically.(Pope and Stevens method).

Reagents :-

1) Cupric Chloride - $CuCl_2.2H_2O$ - 27.3 g/lit.
2) Tri Sodium Phosphate - Na_3PO_4. 12 H_2O - 64.5g.
3) Borate Buffer - $Na_2B_4O_7$.10 H_2O - 57.2gms in 1.5 lit. of water, add 100ml 1N HCl. Dilute to 2 liters with water.
4) Cupric – Phosphate Suspention :- 1 vol. of $CuCl_2$ + 2 vol. of Na_3PO_4+ 2 vol. of Borate buffer and mix.
5) Thymolphthalein indicator
6) Std. N/100 Sod. Thiosulphate soln
7) Starch soln. :- 0.5% (preapare fresh)
8) 1% NaOH soln.
9) KI
10) Glacial acetic acid

Procedure :-

1) Pipette out 25ml of TCA extract of the sample (as prepared for TVBN) in 100ml std. Flask)

2) Add 2 drops of Thymolphthalein indicator. Neutralise this TCA acid with 1% NaOH soln. till light blue color appears.

3) Then add 35ml Cupric – Phosphate Suspention. Make the vol. upto 100ml with DW. Mix it properly and filter.

4) Pipette out 20ml of filtrate in 150ml conical flask. Add about 1g of KI and 15ml of glacial acetic acid.

5) Titrate this rapidly with N/100 $Na_2S_2O_3$ till light yellow color.

6) Then add 1ml of starch soln. again titrate with N/100 $Na_2S_2O_3$ till blue color gets disappear. Note down the reading.

Calculation :-

1ml of 0.01 N $Na_2S_2O_3$ = 0.28 mgm of alpha amino nitrogen.

Alpha amino nitrogen = $\frac{0.28 \times V}{\text{Wt of sample}} \times \frac{50}{25} \times \frac{100}{20} \times 100$ mg%

V = Titre value.

7. Sodium Metabisulphite Test (Qualitative)

Malachite green solution:-

Dissolve 200mg malachite green certified by Biological Stain Commission, in water and dilute to 1lit. Discard when visible deterioration occurs.

Test :-

1) Transfer approximately 3.5gms (½ teaspoonful) ground meat to10 x 10 cm sq. of waxed white freezer paper or other impervious white surface.

2) Add 0.5ml malachite green soln. and mix vigorously for 2min. with hardwood tongue blade or spatula, turning mass frequently.

3) Observe color after few min.

4) Dye is decolorized in presence of sulphites. Normal meat becomes blue – green.

8. Determination of Indole in Shrimp

Indole is used as an index of decomposition. Indole formation in shrimp is supposed to be due the action of bacteria such as *Proteus morganii*, *E. coli.* on shrimp protein. The amount of indole produced is proportional to the extent of decomposition. Shrimp can decompose in the absence of indole-producing organisms. Therefore the presence of indole in shrimp definitely indicates decomposition but the absence cannot ensure that the product is free from spoilage.

Indole is extracted with light petroleum from trichloroacetic acid – precipitated shrimp muscle. The extracted indole, soluble in light petroleum, is reacted and re-extracted with Ehrlich's reagent. Indole in the form of a rose indole complex can be determined spectrophotometrically.

Reagents:-

1) Trichloroacetic acid (TCA) :- 6gm of TCA dissolve in 100ml DW .

2) Petroleum ether, Boiling point 40 – 60^{0}C.

3) Ehrlich's reagent:- Dissolve 9gm para-dimethylaminobenzaldehyde in 45ml conc. HCl acid in 250ml volumetric flask and dilute to volume with ethanol.

4) Std. Indole solutions :- Accurately prepare stock solution of 10mg indole in 100ml light petroleum. Use 1:10 dilution (with petroleum) working solution. Refrigerate indole solutions.

Procedure:-

1) Homogenise 40gm shrimp with 80ml ice-cold TCA solution in a warring blender one min. Add 80ml ice-cold light petroleum and blend for one min.

2) Transfer homogenate to 250ml centrifuge bottle and cenrifuge 10min. at 10,000 rpm. Filter supernate through whatman no. 1 paper under suction.

3) Transfer filtrate to 250ml separatory funnel. After the two layers have separated, transfer acid layer (lower) to second 250ml separatory funnel.

4) Wash TCA- denatured protein precipitate separated by centrifugation with 40ml light petroleum and filter as described above.

5) Transfer filtrate to second 250ml separatory funnel already containing TCA layer from first extraction.

6) Shake 1 min. and let 2 layers separate. Transfer lower acid layer to third separatory funnel and extract for third time with 40ml light petroleum .

7) Combine all light petroleum extracts into 1 separatory funnel.

8) Extract indole with exactly 5ml freshly prepared Ehrlich's reagent by vigorously shaking 1 min.

9) The rose indole complex formed is quantitatively transferred to Ehrlich's reagent layer.

10) When layers have separated, transfer lower layer to 1 cm path cell and read at 570nm against reagent blank solution.

11) Prepare standard curve as follows.

12) Accurately measured volumes from 0.5 to 4ml (5 to 40 microgm) stock indole solution (working solution) into 80ml TCA in separatory funnel.

13) Extract indole by procedures described above and construct standard curve.

14) Rose indole complex from indole standard and from TCA- extracted shrimp is stable up to 4 hour.

Calculation:-

With the help of the standard curve the amount of indole present in 40gm shrimp can be determined. Indole content is usually expressed as the amount of indole in microgram per 100gm shrimp muscle. 250 microgram per kilogram is the limit.

B. Microbial Quality asessment

Fish / Shrimp meat is generally subjected to three main types of bacteriological analysis viz.,

- Aerobic Plate Count (to know the total number of bacteria present in each gram of fish / shrimp meat)
- Faecal indicator bacteria (to estimate the numbers of faecal indicator bacteria such as *Faecal Coliforms*, *E. coli* in the fish / shrimp meat)
- Pathogens (to detect the presence of human pathogenic bacteria such as *Salmonella, Vibrio cholerae, Listeria monocytogenes*, *Staphylococcus aureus* in fish / shrimp meat)

The analyst must aseptically collect representative samples to obtain meaningful results. Established sampling plans / procedures must be strictly adhered to as the results based on these small samples will be used to interpret the condition of large consignments. Always use sterile, leak proof containers to collect the samples. Properly label the sample container. Deliver the samples to the laboratory in iced condition (less than 4 p C) as early as possible. Frozen samples should be kept frozen (-18 p C) at all the times and refrigerated samples (less than 4 p C) should not be frozen.

The methods commonly used for the detection of bacteria are based on

- Morphology and Biochemical methods: based on appearance and presence of enzymes (Gram staining, IMVC tests etc).

- Serological Methods: based on antigen-antibody reaction (Serum agglutination test, Latex agglutination test, Lateral flow immunoassay, ELISA etc)
- Nucleic acid based methods: based on differences in the genome / genes (DNA probes, PCR, RT-PCR etc)

1. Aerobic Plate Count (APC)

Aerobic plate count (APC) is the first analysis that is performed to estimate the number of bacteria in shrimp (solid sample), water and ice (liquid sample) and worker's hand, stainless steel tables, floor (surface samples). Other names for APC are Total Plate Count (TPC), Total Bacterial Count (TBC), Standard Plate Count (SPC). Pour plate method is commonly used to estimate the APC.

1.1. APC of shrimp sample:

- Aseptically collect the fish / shrimp and cut them into small pieces using sterile scissors.
- Weigh 50 grams in a sterile petri dish.
- Transfer the 50g of meat to a stomacher bag and homogenize with 450 mL of diluent (Butterfields phosphate-buffered dilution water) using a stomacher blender.
- The resultant homogenized mixture is 1:10 dilution or 10^{-1} dilution which means that 1 gram of food sample is present in 10 mL of the homogenized mixture.
- Pipette 10 mL from the above 10^{-1} dilution to 90 mL of sterile diluent and mix well. This gives 1:100 or 10^{-2} dilution which means that 1 gram of food sample is present in 100 mL of the homogenized material.
- Pipette 10 mL from the above 10^{-2} dilution to 90 mL of sterile diluent and mix well. This gives 1 : 1000 or 10^{-3} dilution which means that 1 gram of food sample is present in 1000 mL of the homogenized material.

- Similarly further dilutions (10^{-4}, 10^{-5} etc) can be made depending upon the anticipated microbial load.
- Arrange six petri plates in 3 rows in duplicate. Label the plates appropriately (indicating the product name/code, media, date and dilution).
- Pipette 1 mL from the respective dilution (use 10^{-2}, 10^{-3}, 10^{-4} dilutions) and transfer to two petri plates (1ml each).
- Add approximately 12 to 15 mL of molten and cooled (45 ± 1°C) Plate Count Agar (PCA) to each plate.
- Immediately mix the sample dilution and agar medium thoroughly and uniformly rotating the plate thrice in the clockwise direction and thrice in the anti-clockwise direction on a flat level surface.
- Allow the agar to set (leave the plates undisturbed for 15 minutes).
- Invert the solidified agar plates and incubate at 35 ± 1°C for 48 ± 2 hours.

1.2. Controls:

Include media control, diluent control and air controls to assess the sterilization process and the suitably of the work area for bacteriological analysis.

- For media control, pour 12 to 15 mL of molten and cooled PCA in a sterile petri plate.
- For diluent control, transfer 1mL of sterile Butterfields phosphate-buffered dilution water to a sterile petri plate and add 12 to 15 mL of molten and cooled PCA.
- For air control, leave an empty sterile petri plate open on the work table during the period of analysis for 15 minutes and add 12 to 15 mL of molten and cooled PCA. Allow the agar to set (leave the plates undisturbed for 15 minutes), invert the solidified agar plates and incubate at 35 ± 1°C for 48 ± 2 hours.

1.3. Counting of colonies and recording result:

- After the desired incubation period, the colonies developed in each plate are counted using a Qubec colony counter.
- Count all colonies including those of pinpoint size.
- The colony counts of duplicate plates within the same dilution should agree within 10% limit and counts between different dilutions should agree decimally.
- Record the results of each plate dilution wise as shown below.

Dilution	Petri Plate 1	Petri Plate 2
10^{-2}		
10^{-3}		
10^{-4}		

1.4. Calculating APC:

- Select only those plates containing 25 to 250 colonies per plate for calculation purpose.
- Plates with colony counts outside the normal 25 to 250 range may give erroneous indication of the actual bacterial population of the shrimp/fish sample.
- In plates with counts of less than 25, the dilution factors may exaggerate low counts giving very high counts.
- In crowded plates with more than 250 colonies, it may be difficult to count or may inhibit the growth of some bacteria due to limitation of nutrients thus giving very low counts.

APC is calculated using the following formula

$$N = \frac{\Sigma C}{[(1 \times n_1) + (0.1 \times n_2)] \times d}$$

Where,

N = Number of colony forming units per gram of the sample (cfu/g)

ΣC = sum of all the colonies on the selected plates (plates with 25 to 250 colonies)

n_1 = number of plates in the first dilution counted

n_2 = number of plates in the second dilution counted

d = dilution from which the first counts were obtained.

2. Most Probable Number (MPN) method for estimation of Total Coliforms, Faecal Coliforms and *E. coli* in shrimp samples

Total coliforms, faecal coliforms and *E. coli* are used as faecal indicators and their presence indicates poor sanitary condition in the shrimp processing environment. MPN method can be used to detect coliforms and *E. coli* in shrimp samples provided the expected numbers of bacteria are less than 100 per gram; otherwise, the results may not be meaningful. MPN is only a statistical approximation of the test bacteria in the given sample and not the actual number. From the number and distribution of positive and negative reactions, the MPN of the test bacteria in the shrimp sample can be estimated by reference to statistical tables (MPN tables).

Coliforms are Gram-negative, facultatively anaerobic bacteria. They produce gas from glucose and ferment lactose to acid and gas within 48 hours at 35 ± 0.5°C. The coliform group includes species from the genera *Escherichia, Klebsiella, Enterobacter* and *Citrobacter*. Faecal coliforms are defined as coliforms that ferment lactose in EC medium with gas production within 48 hours at 44.5 ± 0.2°C, hence they are referred as thermotolerant coliforms.

- Prepare 10^{-1} (0.1 g per ml), 10^{-2} (0.01g per ml) and 10^{-3} dilutions of the shrimp using Butterfields phosphate-buffered dilution water as diluent. The method for preparing dilutions was previously described in the Aerobic Plate Count section (1.1).

2.1. Step I: Test for Total Coliforms:

- Media: Autoclave Lauryl tryptose broth (LST broth), 10 mL each in 9 test tubes; place inverted Durham's tube in each tube.
- Arrange LST tubes in triplicate sets in a test tube stand. Inoculate 1 mL aliquots (0.1g) from 10^{-1} dilution into 3 LST tubes. Label the tubes as 0.1g.
- Similarly, from 10^{-2} dilution, inoculate 1 mL (0.01g) into three LST tubes and label them as 0.01g.
- Likewise, from 10^{-3} dilution, inoculate 1 mL (0.001g) into three LST tubes and label them as 0.001g.
- Incubate all the LST tubes at 35± 0.5°C.
- Examine tubes after 24 ± 2 hours for gas production. If gas bubble is noticed in inverted Durham's tube, note the reaction as positive.
- Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes.
- Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 3 hours.
- Perform confirmed test on all presumptive positive (gas) tubes.

2.2. Step II: Confirmed Test for Total Coliforms:

- Media: Autoclave Brilliant Green Lactose Bile Broth (BGLB 2%), 5 mL each in test tubes; place inverted Durham's tube in each tube
- The number of BGLB tubes required depends on the number of positive LST tubes in Step I.

- Inoculate loopful of culture from the positive LST tubes to BGLB broth and mark the corresponding label (either 0.1g or 0.01g or 0.001g).
- Incubate BGLB tubes at 35°C± 0.5°C.
- Examine for gas production after 48 ± 3 hours of incubation.
- Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1g, 0.01g and 0.001g).
- Give the result as Total Coliforms MPN per gram.

2.3. Step III: Test for Faecal Coliforms:

- Media: Autoclave EC Broth, 5 mL each in test tubes; place inverted Durham's tube in each tube.
- The number of EC tubes required depends on the number of positive BGLB tubes in Step II.
- Inoculate loopful of culture from the positive BGLB broth to EC broth and mark the corresponding label (either 0.1g or 0.01g or 0.001g).
- Incubate EC tubes at 44.5 ± 0.2°C.
- Examine tubes after 24 ± 2 hours for gas production. If gas bubble is noticed in inverted Durham's tube, note the reaction as positive.
- Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 hours.
- Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g tubes.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1g, 0.01g and 0.001g).

- Give the result as Faecal Coliforms MPN per gram.

2.4. Step IV: Test for E.coli:

- Media: Levine's Eosin Methylene Blue (L-EMB) agar, Nutrient agar slants, Tryptone broth (5ml in test tubes), MR-VP broth (5ml in test tubes), Koser's citrate (5ml in test tubes) or Simmons citrate agar slants.
- Melt EMB agar, cool to 45°C, pour 12 to 15 mL in sterile plates and allow the agar to set.
- Dry the plates in a laminar flow chamber for 45 minutes.
- Streak loopful of culture from the positive EC broth tubes on EMB agar plate and mark the corresponding label (either 0.1g or 0.01g or 0.001g).
- Incubate EMB agar plates tubes at 35°C± 0.5°C for 18-24 hours.
- Observe the EMB agar plates for *E. coli* colonies.
- *E. coli* colonies appear with a greenish metallic sheen by reflected light and dark centered flat colonies by transmitted light.
- Pick suspicious colonies and transfer to PCA slants and incubate at 35 ± 0.5 p C for 18-24 hours.
- Label the nutrient agar slants appropriately (0.1g, 0.01g and 0.001g).
- Perform Gram stain. All the cultures appearing as Gram-negative short rods are further tested by performing IMVC tests (Indole test, Methyl red test, Voges-Proskauer test and Citrate test).
- Indole test: Inoculate the suspected *E. coli* culture from PCA slant into tryptone broth and incubate at 35 ± 0.5°C for 24 ± 2 hours. Test for the presence of indole by adding 0.2 to 0.3 mL of Kovac's indole reagent. Appearance of pink colour in the upper layer (pink ring) is positive. Yellow colour is negative.
- Methyl red test: Inoculate the suspected *E. coli* culture from PCA slant into MRVP broth and incubate at 35 ± 0.5°C for 48± 2 hours. Add 5 drops of

methyl red reagent indicator to each tube. Appearance of distinct red colour is positive. Yellow colour is negative.

- Voges-Proskauer test: Inoculate the suspected *E. coli* culture from PCA slant into MR-VP broth and incubate at 35± 0.5°C for 48 ± 2 hours. To 1mL of the incubated culture add 0.6 ml of á-naphthol solution (0.25g á-naphthol in 5ml ethyl alcohol) and 0.2 ml of 40% KOH (2g KOH in 5ml distilled water). Add a few crystals of creatine. Shake and let it stand for 2 hours. Development of Eosin pink colour indicates a positive test.
- Citrate test: Inoculate the suspected *E. coli* culture from PCA slant into Koser's citrate media tubes (inoculate lightly to avoid detectable turbidity) or streak on Simmon's citrate agar slants and 35±0.5°C for 96 hours. Development of distinct turbidity in Koser's citrate medium or development of blue colour in Simmons citrate agar indicates a positive test.
- The cultures that are Indole positive, Methyl red test positive, Voges-Proskauer test negative, and citrate test negative i.e., IMVC pattern ++— are confirmed as *E.coli* (biotype 1). *E. coli* biotype 2 gives -+— IMVC pattern.
- Note the result as number of positives in each set of 0.1g, 0.01g and 0.001g labelled EMB agar plates.
- Calculate MPN per gram from the 3 tube MPN table (Table for 3 tubes each at 0.1g, 0.01g and 0.001g).
- Give the result as *E.coli* MPN per gram.

2.5. Three Tube MPN Table for food samples

(For 3 tubes each at 0.1g, 0.01g and 0.001g)

Number of Tubes giving a Positive Reaction			MPN per gram
3 x 0.1g	3 x 0.01g	3 x 0.001g	
0	0	0	**< 3.0**
0	0	1	**3.0**
0	1	0	**3.0**
0	1	1	**6.1**
0	2	0	**6.2**
0	3	0	**9.4**
1	0	0	**3.6**
1	0	1	**7.2**
1	0	2	**11**
1	1	0	**7.4**
1	1	1	**11**
1	2	0	**11**
1	2	1	**15**
1	3	0	**16**
2	0	0	**9.2**
2	0	1	**14**
2	0	2	**20**
2	1	0	**15**
2	1	1	**20**

Number of Tubes giving a Positive Reaction			MPN per gram
3 x 0.1g	3 x 0.01g	3 x 0.001g	
2	1	2	**27**
2	2	0	**21**
2	2	1	**28**
2	2	2	**35**
2	3	0	**29**
2	3	1	**36**
3	0	0	**23**
3	0	1	**38**
3	0	2	**64**
3	1	0	**43**
3	1	1	**75**
3	1	2	**120**
3	1	3	**160**
3	2	0	**93**
3	2	1	**150**
3	2	2	**210**
3	2	3	**290**
3	3	0	**240**
3	3	1	**460**
3	3	2	**1100**
3	3	3	**>1100**

2.6. Determining coliforms employing solid media

- Media : Violet Red Bile Agar (VRBA).
- Prepare 10^{-1} dilution of the shrimp sample as described in Aerobic Plate Count method.
- Transfer 1 mL aliquots from 10^{-1} dilution to two sterile petri plates (1ml to each plate).
- Pour 10 mL of VRBA cooled to 48°C into plates, swirl plates to mix, and leave them undisturbed for 15 minutes to allow the agar to solidify.
- Overlay with 5 mL of molten and cooled VRBA to prevent surface growth and spreading of colonies.
- Allow the agar to solidify.
- Invert solidified plates and incubate at 35°C for 18-24 h.
- Examine plates under magnifying lens and with illumination.
- Counting of Coliforms: Count purple-red colonies that are 0.5 mm or larger in diameter and surrounded by zone of precipitated bile acids.
- Confirmation test for coliforms. To confirm that the colonies are coliforms, pick at least 10 representative colonies and transfer each to a tube of BGLB broth. Incubate tubes at 35°C. Examine at 24 and 48 h for gas production. All cultures that produce gas are confirmed as Coliforms.
- ***Note:*** *If gas-positive BGLB tube shows a pellicle, perform Gram stain to ensure that gas production was not due to Gram-positive, lactose-fermenting bacilli.*

3. Detection of Salmonella in shrimp meat:

3.1. Sampling plan for Salmonella (USFDA-BAM)

	Food Category I.	**Food Category II**	**Food Category III**
Definition	Foods that would **not** normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption and are intended for consumption by the **aged, the infirm, and infants**.	Foods that would **not** normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.	Foods that would normally be subjected to a process lethal to *Salmonella* between the time of sampling and consumption.
Analytical unit	Weight of one **Analytical unit = 25g**; Take 25 g analytical unit from **100 g sample unit**.		
Number of analytical units to be tested for each food category	60 analytical units	30 analytical units	15 analytical units
Composite unit	To reduce the analytical workload, the analytical units may be composited. Individual 25g Analytical Units may be combined as **375g Composite Unit**. The maximum size of a composite unit is 375 g or 15 analytical units		

Minimum number of composite units to be tested for each food category	4 composite units	2 composite units	1 composite unit

Sample plan for Raw frozen shrimps: Raw frozen shrimps products fall under Food Category III. Aseptically draw 15 analytical units each weighing 25 grams from 15 different sample units (weighing minimum 100g). Composite the 25g x 15 samples as a single 375g composite sample.

3.2. Analysis of shrimp meat for Salmonella

- Aseptically cut and weigh 25 g of shrimp sample and put it into sterile stomacher bag. Homogenize with add 225 mL sterile lactose broth using a stomacher blender. (For US consignments: Homogenize the 375g of sample with 3375mL of lactose broth and proceed further for Salmonella testing.
- Aseptically transfer homogenized mixture back into the 500 mL flask (For 375 g sample use 5000 mL flask) and allow it to stand for 60 ± 5 minutes at room temperature.
- Mix well by swirling and determine pH. Adjust pH to 6.8 ± 0.2 if necessary.
- Incubate the sample mixture at 35°C for 24 ± 2 hours.
- Transfer 1 mL to 10 mL of Tetrathionate broth (TTB). Mix well
- Transfer 0.1 mL to 10 mL of Rappaport-Vassiliadis (RV) medium. Mix well.
- Incubate TT broth at 35 ± 2°C for 24 ± 2 h in a water bath
- Incubate RV medium at 42 ± 0.2°C for 24 ± 2 h in a water bath
- Mix and streak a loopful of growth from TTB on Bismuth Sulfite agar (BSA), Hektoen enteric agar (HEA) and Xylose Lysine desoxycholate (XLD) agar. Incubate plates at 35°C for 24 ± 2 h

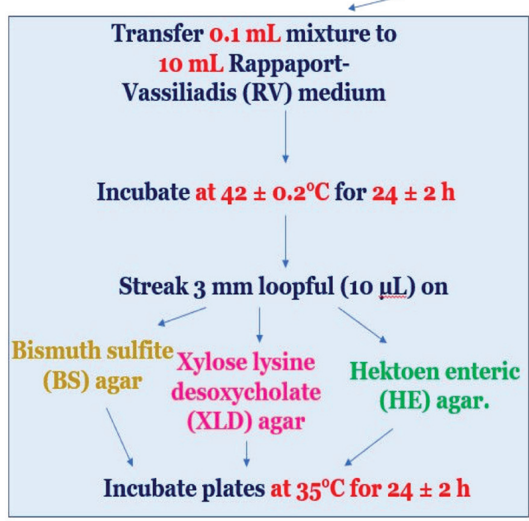
- Mix and streak a loopful of growth from RV medium on Bismuth Sulfite agar (BSA), Hektoen enteric agar (HEA) and Xylose Lysine desoxycholate (XLDA). Incubate plates at 35°C for 24 ± 2 h.
- *Examine all the plates for presence of Salmonella colonies*
- ***Salmonella typical colony*** *appearance on* **Bismuth Sulfite agar (BSA):** Brown, gray, or black colonies ; sometimes they have a metallic sheen. Surrounding medium is usually brown at first, but may turn black in time with increased incubation
- ***Salmonella typical colony*** *appearance on* **Xylose Lysine Desoxycholate (XLDA)**: opaque/ yellow, pink or red with or without black centres
- ***Salmonella typical colony*** *appearance on* **Hektoen enteric agar (HEA)**: Blue-green to blue colonies with or without black centres.
- Pick 2 or more colonies of *Salmonella* from each selective agar. Re-incubate BS agar plates for an additional 24 ± 2 h. Pick 2 or more typical colonies, if present, from the BS agar plates.
- ***Note:*** *In the absence of typical or suspicious Salmonella colonies, search for* ***atypical Salmonella colonies*** *as follows:*
- ***HEA:*** *yellow colonies with or without black centres on HE*
- ***XLD agars:*** *yellow colonies with or without black centres.*
- ***BS agar:*** *Green colonies with little or no darkening of the surrounding medium*
- Lightly touch the very centre of the colony with sterile inoculating needle and inoculate TSI (Triple Sugar Iron) slant by streaking slant and stabbing butt.
- Without flaming, inoculate LIA (Lysine Iron Agar) slant by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm).

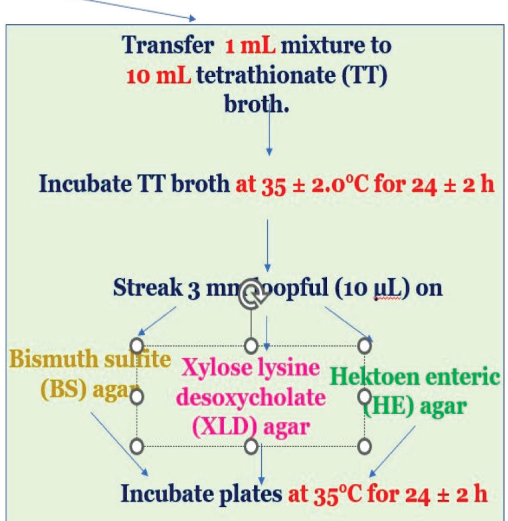
- Incubate TSI and LIA slants at 35°C for 24 ± 2 h.
- **TSI**: Alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (blackening of agar) in TSI.
- **LIA**: alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce H_2S in LIA.
- Retain presumed positive cultures
- ***Note***: All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*.
- Streak TSI agar cultures on MacConkey agar or HEA or XLD agar to obtain pure culture. Incubate plates 24 ± 2 h at 35°C. Examine plates for presence of *Salmonella colonies*.
- Transfer single isolated colony to Nutrient Agar slant
- Perform biochemical and serological identification tests
- Classify, as *Salmonella*, cultures that have reaction patterns of listed in Table below.

SHRIMP

Lactose broth (1:9) (25 g in 225 ml or 375 g in 3375 mL)

Incubate for at 35°C for 24 ± 2 h





3.3. Biochemical and serological reactions of *Salmonella*

Biochemical test	***Test Result***		***Test result for Salmonella species***
	Positive test result	***Negative test result***	
TSI	*blackening Butt*	*non blackening*	Yellow Butt
LIA	*blackening Butt*	*blackening Butt*	*Purple Butt*
Urease	*Purple-red colour*	*No colour change*	*No colour change*
Lysine decarboxylase broth	purple color	yellow color	purple color
Phenol red dulcitol broth	yellow color and/ or gas	no gas; no color change	yellow color and/or gas (*)
Phenol red lactose broth	yellow color and/or gas	no gas; no color change	no gas; no color change (**)

Phenol red sucrose broth	yellow color and/ or gas	no gas; no color change	no gas; no color change
Malonate broth	blue color	no color change	no color change (**)
Indole test	violet color at surface	yellow color at surface	yellow color at surface
Methyl red test	diffuse red color	diffuse yellow color	diffuse red color
Voges-Proskauer test	pink-to-red color	no color change	no color change
Simmons citrate	growth; blue color	no growth; no color change	variable
Polyvalent flagellar test	agglutination	no agglutination	agglutination
Polyvalent somatic test	agglutination	no agglutination	agglutination

* Majority of *S. arizonae* cultures are negative

** Majority of *S. arizonae* cultures are positive

PRACTICAL: Detection of AMR in seafood

A. **Disc diffusion test to detect AMR**(Target bacterium :*Escherichia coli*):

- ***Laboratory Infrastructure*: AST can be performed in laboratories that have basic facilities for microbiological analysis (Laminar flow chamber, autoclave, incubators, weighing balance, pH meter, distilled water unit, glassware, micropipettes, trained personnel)**
- ***Media and chemicals*: Mueller Hinton Agar (MHA), antibiotic discs (selected antibiotics), McFarland Standard-0.5, sterile cotton swabs, ethyl alcohol, sterile normal saline diluents (4ml in glass tubes).**
- ***Bacterial cultures*: Test bacterium and quality control bacterial strain (eg.*E.coli* ATCC 25922).**

The Disc diffusion test is described below as a flow chart.

Isolate and purify the bacterium (*E.coli*) to be tested.

Follow USFDA-BAM (2022, on-line) three tube MPN method using LST broth, BGLB broth, EC broth and EMB agar to quantify and isolate *E.coli* from fish. Confirm the isolate as *E.coli* by performing IMVC tests.

Prepare Mueller-Hinton (MH) agar plates

Accurately weigh required amount of dehydrated MH agar and dissolve it in specified quantity of distilled water as per the manufacturer's instructions. Adjust pH of MH agar using pH meter. Dispense 100 mL quantities of MH agar in 250 mL flasks. Sterilize by autoclaving at 15 psi for 15 minutes. Cool the MH agar (approximately up to 45ºC) and pour MH agar into sterile petri plates. Note that the MH agar plate should have a uniform depth of 4mm±0.5mm. This can be achieved by pouring 25mL of molten MH agar in each 90mm petri plate. Dry the MH agar plates by placing the plates with lids ajar in laminar flow chamber at room temperature for 30 minutes (Drying of plates is done to avoid excess moisture which may result in problems with fuzzy zone edges).

Prepare inoculum of bacteria by employing the direct colony suspension method or growth method*Direct colony suspension method*:

Streak *E.coli* culture on non-selective medium such as nutrient agar or blood agar. Incubate at 35±2ºC for 18h (overnight). Prepare a saline suspension of the *E.coli* culture

by picking four to five colonies with a sterile bacteriological loop and suspending them in sterile normal saline (4mL) so as to obtain 0.5 McFarland turbidity that corresponds to 1.5 x 10^8cfu/ml of *E.coli*. (Visually compare the bacterial suspension and McFarland standard against a white background with black stripes). Adjust turbidity equivalent to 0.5 McFarland standard by adding sterile normal saline or more bacteria. This bacterial suspension is the inoculum for further use. (Standardizing the inoculums suspension is essential as a denser inoculum results in reduced zones of inhibition and less dense inoculums will result in larger zones of inhibition)OR **Growth method**: Streak *E.coli* culture on non-selective medium such as nutrient agar or blood agar. Incubate at 35±2ºC for 18h (overnight). Using a sterile bacteriological loop touch the top of four or five colonies and inoculate into tryptic soy broth or tryptone broth and incubate at 35±2ºC till it reaches a turbidity equivalent to 0.5 McFarland standard. This bacterial suspension is the inoculum for further use. (The inoculum should be used within 15 minutes of preparation)

Inoculate MH agar with the inoculums

Dip a sterile cotton swab in to the tubes containing the inoculum. Press the swab to the inner wall of the tube to remove dripping fluid. Spread the inoculums by swabbing on the surface of MH agar, by moving the swab in front and back motion and at the same time rotating the plate to ensure even distribution of the inoculums. Make a final sweep of the swab around the agar rim. Leave the plates

undisturbed for 3 to 5 minutes to allow the inoculums to be absorbed.

Application of antibiotic discs

Apply the antibiotic discs within 15 minutes of swabbing the inoculums. Select the antibiotic discs impregnated with the appropriate antibiotic concentration. Using a sterile forceps, aseptically remove an antibiotic disc from the container and dispense it evenly on the surface of dried MH agar. Gently press the antibiotic disc to ensure firm contact with the agar surface. (Antibiotic discs should not be placed too near to the rim of the petri plate. Do not move the antibiotic disc after it is applied to the agar surface as the diffusion of antibiotic from the disc is fast and if relocated might give erroneous results). Flame the forceps after dipping in ethyl alcohol and repeat the process for the next antibiotic disc. A maximum of 6 antibiotic discs can be applied on 90mm petri plate so as to avoid overlapping of inhibition zones and interference of different antibiotics. While placing the discs, ensure that wide gap is maintained between different antibiotic discs. (Antibiotic discs are stored in refrigerator (< 8ºC) or freezer (-20ºC) depending on the manufacturer's instructions. One hour prior to application of the antibiotic discs, remove the disc container from the refrigerator/ freezer and allow them to equilibrate to ambient temperature. Examine the disc containers for expiry date as loss of potency of antibiotic present on the disc results in reduced zone diameters leading to wrong results)

Incubation of the inoculated MH agar plates

After inoculation of the bacterial suspension and application of the antibiotic disc, the MH agar plates are inverted and incubated at $35^{o} \pm 2^{o}C$ for 16 to 20 hours in an incubator. Do not stack plates in the incubator as it might result in uneven heating of plates.(Incubation step should be performed within 15 minutes of completion of application of antibiotic discs)

↓

Interpretation of Disc diffusion test result

Observe the MH agar for clear and circular zone of inhibition surrounding each antibiotic disc and the remaining area filled with a confluent lawn of bacterial growth. Measure the diameter of the zone using a ruler or calipers. Place the ruler or calipers on the back of the inverted petri plate and measure the diameter of the zone to the nearest millimeter by holding the plate above a dark background illuminated with reflected light. Zone margin is the area that shows no visible bacterial growth when seen with the naked eye; with the petri plate held 30 cm away from the eye. (Do not use transmitted light or magnifying glass during measurement). Repeat this for all the other antibiotic discs. Record the zone diameters obtained for each of the antibiotic tested.

Interpret the result

Compare the zone diameter by referring to breakpoint tables for the *E.coli* specified by CLSI/EUCAST.

Based on the AST result, bacteria can be classified as susceptible, resistant, or intermediate in response to a specific antibiotic

Susceptible means that the antibiotic is effective against the test bacterium.

Resistant means that the antibiotic is ineffective against the test bacterium.

Intermediate means a higher dose of the antibiotic and/or or a longer period of time is needed to prevent growth of the test bacterium

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Quality control:

- Use recommended quality control reference strains (eg*Escherichia coli* ATCC 25922) to monitor the performance of disc diffusion test. The control strains can be obtained from ATCC or other culture collections. Control strains should be tested regularly and the strains must meet CLSI established/acceptable zone diameters.
- Check the performance of every new batch of Mueller Hinton agar using reference bacterial strains

B. **Minimum inhibitory concentration (MIC) testing employing E-test:** MIC is the lowest concentration of a specific antibiotic that inhibits the growth of a specific bacterium. E-test (Epsilometer test)is a simple and cost-effective method to determine MIC of antibiotics. E-test consists of a predefined, continuous, exponential gradient of a specific antibiotic that is immobilized on a thin inert non-porous plastic carrier strip (E-test strip).

Procedure of E-test: The test procedure is similar to disc diffusion method. The difference is that instead of applying antibiotic disc, in the E-test plastic strip impregnated with a gradient of antibiotic are applied.

The initial steps of preparing the bacterial suspension (inoculums) and swabbing the inoculums MH agar plates are same as described in disc diffusion method.

Using sterile forceps aseptically place the E-test strips on the MH agar surface. Place the E-test strip with the antibiotic concentration scale visible (numbered scale should face upwards while applying the E-test strip on the MH agar surface). Place one E-test strip onto a 90 mm plate. Do not move the E-test strip after it is applied to the agar surface there is an immediate release of the antibiotic and an antimicrobial concentration gradient gets established in the MH agar medium.

After inoculation of the bacterial suspension and application of the E-strip, the MH agar plates are inverted and incubated at 35° ± 2°C for 16 to 20 hours in an incubator.

Read the E-test result by viewing the E-test strips from the top of the petri plate. The zone of inhibition appears as a symmetrical ellipse (drop shaped inhibition zone).

The MIC is read as the lowest concentration of the antibiotic at which the ellipse (drop-shaped inhibition zone) intersects the antibiotic concentration scale marked on the E-test strip. Read the MIC at the point of complete inhibition of all growth including isolated colonies.

↓

If MIC value appears to between two antibiotic concentrations, then record the higher value as MIC. Ignore any growth at the edge of the strip

↓

Interpret the result by comparing the MIC to breakpoint values for the *E.coli* specified by CLSI/EUCAST.

↓

Categorize the bacterium as Susceptible, Intermediate or resistant to the antibiotic tested.