Training manual
on
Seafood Quality Assurance

Under Schedule Caste Sub-Plan (SCSP)

Organized by

ICAR-Central Institute of Fisheries Technology

(20-24 February 2023)

ICAR-Central Institute of Fisheries Technology
Willingdon Island, Matsyapuri P.O.,
Cochin – 682 029 (INDIA)
2023

Published by: Director, ICAR-CIFT, Cochin-682029

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For citing the Training manual:

Priya, E.R., Uchoi, D., Nadella, R.K., Kishore, P., Chatterjee, N. S., Laly S.J., Femeena, H. and Ninan, G. (eds.) (2023) Seafood Quality Assurance. Central Institute of Fisheries Technology, Cochin, India. pp 140.

For citing the chapter

Priya, E. R. and Laly, S. J. Post- mortem changes and mechanism of fish spoilage. In: Priya, E.R., Uchoi, D., Nadella, R.K., Kishore, P., Chatterjee, N. S., Laly S.J., Femeena, H. and Ninan, G. (eds.) (2023) Seafood Quality Assurance. Central Institute of Fisheries Technology, Cochin, India. p. 10-17.

Design and Cover page: Dr. Devananda Uchoi

FOREWORD

Quality of food plays a significant role in the development of healthy life and its safety remains utmost importance in the food production system. Today, lot of concern has been raised globally by consumers on different food safety related issues. Food contamination in the food chain is happening on regular basis and proper identification of hazards associated with the food production systems is the need of the hour as hazards may be introduced at any stage. Food-borne outbreaks have been occurring due to the consumption of contaminated food. Frequent outbreaks have been reported which are caused by different human pathogenic bacteria such as *Salmonella*, *Vibrio parahemolyticus*, *E. coli* O157:H7 and *Listeria monocytogenes* etc. Apart from this contamination is also happening due to the adulteration of food with different chemical substances and also due to residues of pesicides, antibiotics and accumulation of heavy metals as a result of increase in pollution. Proper identification of these contaminants in food is required for mitigating the risks raised due to the emerging contaminants in foods and thereby improving its quality and safety to consumers.

ICAR-Central Institute of Fisheries Technology (CIFT) being a premiere government organization has been continuously working on the quality and safety of fish and fishery products since its inception. In India, ICAR-CIFT was involved in the formulation of several international and national standards pertaining to the quality and safety of fish and fishery products. Apart from this, ICAR-CIFT was the driving force for setting the standards for packaged drinking water in India. A significant achievement in the ICAR-CIFT's relation to the seafood processing sector has been associated with the introduction and implementation of Hazard Analysis and Critical Control Point (HACCP) based quality assurance system in the processing industries throughout the nation. In collaboration with Food Safety Standards Authority of India (FSSAI), ICAR-CIFT is working to set standards for the quality of Fish and Fish products and its consumption domestically. Based on the pioneering work carried out by Central Institute of Fisheries Technology, FSSAI recognised ICAR-CIFT as the National Referral Laboratory as well as National Reference Laboratory for fish and fishery products. ICAR-CIFT has extended its support to FSSAI in formulation of development of food safety standards, food testing protocols and development of code of practise. Technologies developed by the ICAR-CIFT have played an important role in improving the standards of harvest and post-harvest fishery sectors of India, thereby making it as one of the major seafood exporter.

Training programme on 'Seafood Quality Assurance' was organized for college students under ICAR sponsored Scheduled Caste Sub Plan (SCSP) for uplifting the sector and to improve their technical skills.

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PREFACE

Food safety is a global concern due to its direct effect on human's health. Fish and fishery products constitute a significant component of human diet. Contribution of fish and other aquatic products in the average animal protein consumed worldwide is around 15 percent. Fish and other seafood in daily diet is a smart choice for health-conscious consumers. There are proven health benefits of consumption of aquatic products that out-weigh risks. Though fish provide many health benefits, seafood can be compromised by different chemical and biological contaminants which are harmful to consumers, if they are harvested from waters contaminated with industrial chemicals, heavy metals, pesticides antibiotics residues and different human health hazard bacterial pathogens. These contaminants may accumulate in fish at levels that are harmful for human health (e.g. carcinogenic and mutagenic effects). Food can become contaminated with contaminants at any point during production, distribution and preparation. Everyone along the production chain, from producer to consumer, has a role to ensure the safety of seafood. The seafood may get contaminated with various pathogenic bacteria due to unhygienic handling practices, cross contamination of raw foods with cooked or ready-to eat foods, and lack of proper time - temperature control. Bacterial and viral pathogens including parasites which occur naturally are the primary food safety concern with regard to seafood. The vital tools commonly used to define the requirements for an effective Food Safety Management System are ISO 22000 and HACCP (Hazard Analysis and Critical Control Points). These are the basis for Food Safety principles defined by Codex Alimentarius Commission of World Health Organization. HACCP is an internationally recognized risk management tool, which is proactive in nature, while ISO 22000 is a complete food safety management system, enabling continual improvement of performance. The training programme on 'SEAFOOD QUALITY ASSURANCE' is an attempt to give a clear picture to the participants specifically on the aspects of mechanism of spoilage in fish, sensory evaluation of seafood, different hazards in seafood, methods of detection, different analytical methods for detection of contaminants, quality issues in seafood, preservation methods of seafood, chemical and microbiological quality indices, fundamentals of bacteriology, major seafood borne pathogens and its detection techniques, sanitation and hygiene requirements in seafood processing industries, analysis of histamine, antibiotic and pesticide residues, HACCP in food safety management system and chromatographic techniques. We would like to thank Indian council of Agricultural Research, New Delhi for providing opportunity to conduct this training programme and also for fund allocation under SCSP. We acknowledge the entire resource persons for immensely contributing for this manual. In fact the entire manual was prepared during the training programme itself and without the support of all faculties it would not have been possible. We would like to acknowledge the programme director, Dr George Ninan, Director, ICAR-CIFT for all the support, guidance and encouragement given for the successful completion of this training programme as well as training manual. We hope that this publication will serve as a guide for academicians and students to understand the concepts of seafood quality assurance.

Course Directors &
Course Coordinators

Content

Chapter 1

Overall Perception of Fish Safety and Quality

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Safety is an essential pre-requisite for quality and represents the minimum standard that guarantees seafoods on the hygienic-sanitary point of view. A further safety tool is represented by the product traceability and labelling that supply detailed and precise information to the chain operator, only some of them reaching consumers: identification number of each lot, identification number and name of the fishing vessel or name of the aquaculture production unit, date of catches or the date of production, quantities of each species (kg or no of individuals), name and address of suppliers, scientific name and commercial designation of species, relevant geographical area, production method (farmed or captured) and whether the fisheries products have been previously frozen or not (Reg. CE 1224/2009). Ethical aspects too are assuming an increasing importance for consumers, when they are sensitive to the fact that seafood they will use were obtained with sustainable fishery/aquaculture systems and with respect for animal welfare.

Product quality evaluation starts from a careful examination of the external aspect of the species under interest, on the basis of the distinctive features such as skin or carapace or valve colour, and morphological traits of commercial interest. Proper morphology and merchantable traits are evaluated through a series of length and weight measures. Length measures also have an important role at commercial level for the main species. A minimum size, below which fishing and marketing are not allowed, was fixed for each main species (Reg. EC 1967/2006, Annex III). This was decided because the smaller specimens, still juveniles, must be protected to assure a sustainable exploitation of resources. For commercial size fish the measures to be pointed out are gutted weight/ fillet yields, and condition factor. Condition factor (body weight/length ratio) indicates the fish corpulence within species, often related to body and meat adiposity. Apart from the feeding history, some quality aspects can also differ according to size because with increase in fish body weight and age, muscle and mesenteric fat incidences increase, while the one of bony tissue decreases. The fish reared in floating cages generally show less fat, both in viscera and in fillet, and better sensorial quality, in comparison to the ones reared in tanks. The different nutritional state, the higher energetic

consumption/swimming activity and the streamlined flow in the cage are at the basis of the main differences, making them similar to the product captured in the wild. The chemical, nutritional and dietetic characteristics, peculiar of the species but markedly influenced by extrinsic parameters, such as quantity, quality and feeding modality.

The physical and organoleptic characteristics can be evaluated through the behaviour of rigor mortis phases (pre-rigor, full rigor, rigor release), the changes of dielectric properties (indices of fish integrity loss), pH, colour, texture and freshness/quality state. Freshness state evaluated by sensorial methods through examination of the general aspect of eyes, skin, gills, odour of gills, flesh texture, resilience and colour on the raw product, and flesh texture, colour, taste, flavour and juiciness on the cooked product is able, even alone, to be a reliable index of seafood quality. Evaluation methods more frequently used are the ones officially accepted in Europe (Reg. EU 2406/1996) distinguishing three freshness classes, very fresh (Extra), fresh (A), bad quality (B), below B fish being discarded for human consumption, or the Quality Index Method, a specific demerit index that assumes 0 value in very fresh fish, increasing value with quality worsening. In the case of reared product, freshness state of each species could even be estimated from the harvesting date, when a correct and uninterrupted cold chain has been assured. The time period in which seafood is marketable (shelf life) could also be evaluated both by sensorial methods, and as total viable count (TVC) or charge of individual specific spoilage organism (SSO), the latter are the ones better developing at the selected conservation conditions (for example *Pseudomonas* in refrigerated product, *Photobacterium* in Modified Atmosphere Packed, MAP product). Raw product is considered unfit for human consumption according to the sensorial parameters days before in comparison to the edibility threshold indication as TVC (10^7 cfu/g), the later resulting more fit as spoilage index in cooked product. Among the physical traits instrumentally determined, some of them are to be mentioned: skin and fillet colour, important for fish with pigmented flesh- evaluated (CIELab system) through the colorimetric parameters lightness (L*), redness (a*) and yellowness (b*), chroma and hue - and texture, important both as product sensorial aspect and for processing attitude. Texture increases with the muscular fibre density and diameter, and with the quantity and ageing of collagen structure. At the same weight, wild fish generally have flesh more consistent than those of cultured ones, also for their lower fat quantity and the greater muscular tissue activity for swimming. Texture decreases as fish freshness declines, and can be considered as a non-destructive index of freshness. Other useful aspects for the instrumental evaluation of quality

changes in the final phases of shelf life are the levels of biogenic amine (histamine, putrescine, cadaverine) of malonaldehyde, secondary lipid oxidation compounds and odour volatile compounds.

Major developments in the field of fish safety and quality have had a significant impact on international trade during the last few decades. Technological developments in fish handling and processing coupled with increasing consumer food safety and quality awareness have resulted in the adoption of HACCP-based systems and scientifically-based risk assessment methodologies. This is reflected in international regulatory framework of the SPS and TBT Agreements of the WTO and the normative work of CODEX Alimentarius. Based on available food borne illness reports, the decrease in food borne diseases has coincided with the implementation of HACCP-based food safety assurance measures. However, fish safety and quality issues related to indigenous microorganisms, chemical or veterinary drugs are increasingly of concern. This reflects the need for a food chain approach in the analysis of hazards and risks to develop integrated risk management strategies. However, a food chain approach also requires substantial multidisciplinary scientific information given the need of science-based risk analysis.

FAO provides direct assistance to member countries via the CODEX Committees and other expert groups with a focus on training and capacity building in developing countries. In response to increasing demands for pertinent and succinct scientific and technical information upon which to conduct adequate hazard and risk analysis, the FAO has launched the Aquatic Food Programme in collaboration with the Canadian Food Inspection Agency with the expectation of creating a peer reviewed comprehensive knowledge base of integrated aquatic food to safety and quality information from a food chain approach. Safety and quality concepts are incorporated in the FAO Code of Conduct for Responsible Fisheries, particularly Articles 6 and 11.

ISO 8402: 1995 standard defines general quality as: “the totality of features and characteristics of a product or service that bears its ability to satisfy stated or implied needs.” The ISO 9000 standard quality system modified the quality concept, shifting the attention from the final product to all the processes contributing to its production. This integrated management system approach, in which planning, personnel involvement, documentation of activities and the attitude towards a continuous enhancement became the basis of the new management model. This is the background of the total quality concept.

In a fishery chain, total quality could be defined as the complex of the characteristics able to satisfy the organoleptic, health, use/price convenience requirements of the purchaser/consumer, constantly found and obtained through a correct management of the production chain, with respect of welfare and environment sustainability, and made known in full transparency through traceability and labelling.

Practical implementation of international regulatory frameworks commenced in the early 1980s when many countries engaged in reforming their fish inspection systems to implement preventive HACCP-based safety including quality systems and supporting hygiene and sanitary requirements. While there is growing and strong evidence that the implementation of HACCP based systems has contributed to improve fish safety and quality significantly, there has been recent growing awareness of the importance and need of an integrated, multidisciplinary approach to safety and quality, considering the entire fish food chain. FAO defines the food chain approach as recognition that the responsibility for the supply of food that is safe, healthy and nutritious is shared along the entire food chain by all involved with the production, processing, trade and not the least the consumption of food. Stakeholders include farmers, fishermen, food processors, transport operators, distributors, consumers, as well as governments obliged to protect public health. This holistic approach to food safety along the food chain differs from previous models in which responsibility for food safety concentrated mainly on the food-processing sector and government control services. The implementation of a food chain approach requires an enabling policy and regulatory environment at national and international levels with clearly defined rules and standards, establishment of appropriate food control systems and programmes at national and local levels, and provision of appropriate training and capacity building (FAO, 2003). Efforts to integrate these developments into fish safety and quality policies are ongoing at national, regional (e.g., European Union, EU) and international (e.g., Codex Alimentarius Commission) levels. Fish safety regulators have been applying a host of control measures, from mandating the use of HACCP to increasing testing, with varying degrees of success. However, the various scientific tools available to support the development of a food chain approach present limitations, which needs to be recognized and considered, including gaps in research data. Indeed, much of the data needed to develop science-based strategies are often incomplete, non-existent or require extensive resources to generate. In addition, the link between the food safety criteria and public health objectives is not always present in current safety regulations. Consequently, improved scientific tools must be adopted and novel approaches must be sought so that the need for regulatory control can be

balanced with the need for regulatory flexibility and with the expectation that a regulatory agency's actions reflect the most current and effective scientific methods available to protect the public health.

In the fish industry, there are five broadly defined needs on which a strategy in support of a food chain approach to food safety should be based:

- Fish safety and quality from a food chain perspective should incorporate the three fundamental components of risk analysis–assessment, management and communication–and, within this analysis process; there should be an institutional separation of science-based risk assessment from risk management–which is the regulation and control of risk.
- Traceability from the primary producer, through post-harvest treatment, processing and distribution to the consumer must be improved.
- Harmonization of fish quality and safety standards, implying increased development and wider use of internationally agreed, scientifically based standards is necessary.
- Equivalence in food safety systems–achieving similar levels of protection against fish-borne hazards and quality defects whatever means of control are used–must be further developed.
- Increased emphasis on risk avoidance or prevention at source within the whole food chain–from farm or sea to plate–, including development and dissemination of good aquaculture practices, good manufacturing practices and safety and quality assurance systems (i.e., Hazard Analysis and Critical Control Point [HACCP]), are necessary to complement the traditional approach to fish safety and quality management based on regulation and control.

The principles of achieving harmonization of standards and equivalency in food control systems and the use of scientifically based standards are embodied in the two binding agreements of the WTO (the Agreement on the application of sanitary and phytosanitary [SPS] measures and the Agreement on technical barriers to trade [TBT]). The SPS Agreement confirms the right of WTO member countries to apply measures necessary to protect human, animal and plant life and health. The purpose of the SPS Agreement is to ensure that measures established by governments to protect human, animal and plant life and health, in the agricultural sector, including fisheries, are consistent with obligations prohibiting arbitrary or unjustifiable discrimination on trade between countries where the same conditions prevail and are not disguised restrictions on international trade. It requires that, with regard to food safety

measures, WTO members base their national measures on international standards, guidelines and other recommendations adopted by the Codex Alimentarius Commission (CAC) where they exist. This does not prevent a member country from adopting stricter measures if there is a scientific justification for doing so or if the level of protection afforded by the Codex standard is inconsistent with the level of protection generally applied and deemed appropriate by the country concerned. The SPS Agreement states that any measures taken that conform to international Codex standards, guidelines or recommendations are deemed to be appropriate, necessary and not discriminatory. Finally, the SPS Agreement requires that SPS measures are to be based on an assessment of the risks to humans, animal and plant life using internationally accepted risk assessment techniques. The objective of the TBT Agreement is to prevent the use of national or regional technical requirements, or standards in general, as unjustified technical barriers to trade. The agreement covers standards relating to all types of products including industrial products and quality requirements for foods (except requirements related to SPS measures). It includes numerous measures designed to protect the consumer against deception and economic fraud. The TBT Agreement basically provides that all technical standards and regulations must have a legitimate purpose and that the impact or cost of implementing the standard must be proportional to the purpose of the standard. It also states that, if there are two or more ways of achieving the same objective, the least trade restrictive alternative should be followed. The agreement also places emphasis on international standards, WTO members being obliged to use international standards or parts of them except where the international standard would be ineffective or inappropriate in the national situation. The aspects of food standards that TBT requirements cover specifically are quality provisions, nutritional requirements, labeling, packaging and product content regulations and methods of analysis. Unlike the SPS Agreement, the TBT Agreement does not specifically name international standard setting bodies, whose standards are to be used as benchmarks for judging compliance with the provisions of the Agreement. Risk analysis is widely recognized today as the fundamental methodology underlying the development of food safety standard that provides adequate health protection and facilitates trade in food (FAO, 2001). There is a fundamental difference between a hazard and a risk. A hazard is a biological, chemical or physical agent in, or condition of food, with the potential to cause an adverse health effect. In contrast, risk is an estimate of the probability and severity in exposed populations of the adverse health effects resulting from hazard(s) in food. Risk analysis is a process consisting of three components: risk assessment, risk management and risk communication. Risk assessment is the scientific

evaluation of known or potential adverse health effects resulting from human exposure to food-borne hazards. Risk management is the process of weighing policy alternatives to accept, minimize or reduce assessed risks and to select and implement appropriate options. Risk communication is an interactive process of exchange of information and opinion on risk among risk assessors, risk managers and other interested parties. The responsibility for the supply of fish that is safe, healthy and nutritious should be shared along the entire chain from primary production to consumption. Development and implementation of Good Aquaculture Practices (GAP), Good Hygienic Practices (GHP) and Hazard Analysis Critical Control Point (HACCP) are required in the food chain step(s). Government institutions should develop an enabling policy and a regulatory environment, organize the control services, train personnel, upgrade the control facilities and laboratories and develop national surveillance programs for relevant hazards. The support institutions (academia, trade associations, private sector, etc.) should also train personnel involved in the food chain, conduct research on quality, safety and risk assessments and provide technical support to stakeholders. Finally, consumers and consumer advocacy groups have a counter balancing role to ensure that safety and quality are not undermined by political considerations solely when drafting legislation or implementing safety and quality policies. They also have a major role in educating and informing the consumer about the major safety and quality issues. The general principles of GHP/HACCP were adopted by the Codex Alimentarius Commission (CAC) in 1997 and 1999 (FAO, 2001). They include requirements for the design and facilities, control of operations (including temperature, raw materials, water supply, documentation and recall procedures), maintenance and sanitation, personal hygiene and training of personnel. Similarly, the Codex Committee on Fish and Fishery products is working on a draft code of practice for fish and fishery products, including aquaculture products, which integrates these general principles and adapts them to aquaculture. However, this Code is not intended to cover extensive fish farming systems or integrated livestock and fish culture systems that dominate production in many developing countries. Control and prevention of chemical pollutants and biotoxins require the implementation of appropriate monitoring and surveillance programs. This is particularly important for mollusc culture, filter feeders that can concentrate pollutants, biological agents and biotoxins. The Codex Code of Practice describes the requirements for surveys and monitoring of the harvesting and growing areas to determine sources of domestic and industrial pollution, classification of the areas into suitable for harvesting, relaying or non-suitable for growing or harvesting, and the frequency and methods of monitoring.

Fish Safety and Quality Knowledge Base Fish and seafood are produced from a great variety of plant and animal aquatic species. A risk analysis focused on a specific hazard such as a pathogen or a contaminant requires a substantial amount of scientific and technical information. Each species have different safety and quality attributes related to local conditions and production methods in addition to the type of food commodity, which also has specific processing and preservation requirements. Better utilization of aquatic resources and the harmonization of fish safety and quality systems require access to updated scientific and technical information not the least in light of the SPS Agreement of the WTO that require science-based risk analysis of food hazards. Over the years, FAO has experienced this first hand in risk assessment exercises such as *Listeria monocytogenes* or Vibrio spp. These also proved to be cost and resource intensive given the often-lengthy time frames and the number of experts involved. Since the late 1990s, FAO has been aware of the need for integrated and succinct technical information. The current thinking of a food chain approach to safety and quality simply exacerbates this need. In addition to potential food hazards that may be introduced via handling and processing, fish and seafood production methods include the fisheries of wild populations and aquaculture where safety and quality also depends on the local conditions of the environment and habitat. Given FAO's normative work and capacity building mandate in developing countries, FAO is launching the Aquatic Food Programme in collaboration with the Canadian Food Inspection Agency and other international organizations. Although the Internet offers a wide range of scientific information, finding adequate and pertinent information can be perplexing for a novice user of the Internet. In addition, today's electronic information dissemination capabilities are less of a challenge than the work involved in updating information. The understanding and integrated management of risks along the entire food chain requires substantial integration of technical information based on the latest available scientific literature and knowledge.

Fishery products are the most traded food in the world. The globalization and further liberalization of world fish trade presents new safety and quality challenges. Upgraded scientific tools must be implemented and novel flexible methods to safety must be sought so that regulatory actions can reflect the most current scientific evidence, and this in turn helps to share the responsibility for safety among the stakeholders of food chain. Fish safety and quality assurance will require enhanced levels of international co-operation in promoting harmonization, equivalency schemes and standards setting mechanisms based on science. The SPS/ TBT agreements of the WTO and the benchmarking role of the Codex provide an

international platform in this respect. Important reforms to tackle these issues have been initiated in the USA (NAS, 2003), the EU (2000) and many other countries. Unfortunately, developing countries are at a disadvantage because of insufficient/inadequate national capacities and resources. International organizations such as FAO must revamp their programmes and seek the necessary resources to assist in this endeavour.

Chapter 2

Post- Mortem Changes and Mechanism of Fish Spoilage

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Products with no proven quality find little space in the market. The term quality represents the consumer acceptability of the commodity. The eating quality of fish determines the acceptability of fish as food to the consumer. The quality of fish begins to deteriorate immediately after catch. The changes taking place in fish after death are collectively called "post-mortem changes" and they can be grouped as follows:

- Hyperaemia
- Rigor Mortis
- Autolysis
- Microbial putrefaction/decomposition
- Lipid oxidation/autoxidation
- Discoloration

Hyperaemia:

During the harvest, the fish passes through various levels of struggle due to asphyxia or unfavorable conditions. At this time, the mucus glands in the skin secrete a large quantity of mucus, forming a thick layer of slime on the skin's surface. This phenomenon is known as "Hyperaemia". The slime contains an antimicrobial substance lysozyme which protects the fish from the attack of spoilage bacteria. After death, the immunological properties are lost, and microbes easily proliferate on the glycoprotein content of the slime imparting an offensive smell and increasing the penetration further into the muscles and internal organs.

Rigor mortis:

After the capture till the point of consumption, the fish undergoes a large number of physicochemical changes, which can be classified into three stages:

1. *Re-rigor state:* It is the state of fish from death till the onset of rigor mortis. At this stage, the fish is extremely fresh and the muscles remain elastic. This state is characterized by a fall in ATP and creatinine phosphate and glycolysis.
2. *Rigor mortis:* It is the state of fish from the onset of rigor mortis till its disappearance. At this stage the fish muscles become stiff. The stiffening of muscle after death is called

"rigor mortis". It starts 1-7 h after death, reaches a peak between 5-22 h, and its total duration range from 31-120 h.

3. *Post-rigor state:* It is the state of fish after the disappearance of rigor mortis. During this stage, the meat tenderization takes place making the fish organoleptically acceptable.

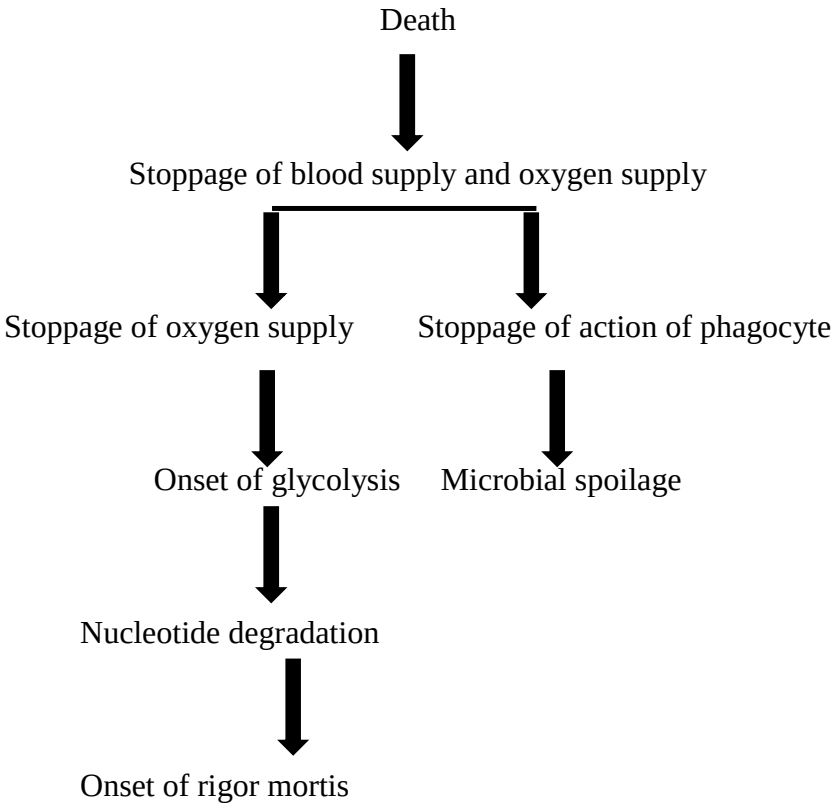
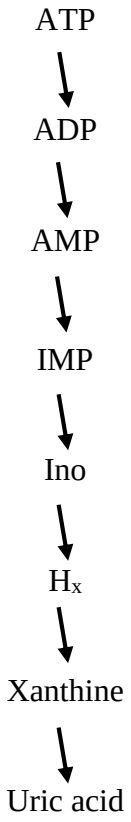


Fig1. Post-mortem changes of fish

Post-mortem Glycolysis: On cessation of oxygen supply to the muscles, the main carbohydrate source, glycogen broken down to lactic acid by anaerobic pathways -hydrolytic/amylolytic pathway & phosphorolytic pathway. As lactic acid accumulates in the system, the pH of the system falls from the initial physiological pH of 7.2-7.4 to the ultimate post-mortem pH of 6.0-6.2.

Nucleotide degradation: Nucleotide degradation is one of the earliest indices to assess freshness. It reflects both the action of autolytic enzymes and bacterial action. The nucleotide degradation products – Inosine Monophosphates (IMP), Hypoxanthine (H_x) or K value clearly reflect the quality loss in fish. After the death of the fish, Adenosine triphosphate (ATP) is degraded by endogenous enzymatic action and forms Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), IMP, Inosine (Ino), and H_x successively. Hypoxanthine is

further degraded by xanthine oxidase to xanthine and uric acid. The degradation of ATP up to IMP is very fast, but the degradation of IMP is relatively slow. IMP imparts a pleasant, sweet taste and flavor (Umami taste, especially in crabs. Degradation of IMP to inosine and hypoxanthine results in a bitter taste and progressive loss of desirable flavor. The sequence of nucleotide catabolism in fish is given below:



Autolysis:

The process of breakdown of fish tissue after death by endogenous enzymes is known as "Autolysis". The breakdown of proteins, lipids and nucleic acids into their simpler units of significance during autolysis, results in softening of the tissue.

Microbial putrefaction/decomposition:

Autolysis alone will not spoil fish but helps the spoilage process by providing a nutrient-rich medium to the microbes. Microbes secrete their own enzymes to hydrolyze tissue components into simpler substances. Microbial putrefaction is the process of conversion of tissue components by microorganisms into off-odor and off-flavor substances to make the fish spoil.

Lipid oxidation/autoxidation:

Lipid oxidation is the limiting factor in fatty fish during storage, which results in rancidity (development of off-flavor and off-odor). The factors affecting the onset and development of rancidity are

1. Degree of unsaturation
2. Type and concentration of antioxidants
3. Pro-oxidants
4. Moisture content
5. Oxygen availability
6. Temperature
7. Degree of exposure to light

The major chemical indicators for the determination of the extent of oxidative rancidity are anisidine value (AV), peroxide value (PV), and thiobarbituric acid value (TBA). Peroxide value is also known as hydroperoxide value, used as a measure of the extent of oxidation in the early stages. It measures the primary products of lipid oxidation, which break down into secondary products of oxidation or reacts with protein. An increase in PV is most useful as an index of the earlier stages of lipid oxidation; as the oxidation proceeds the PV starts to fall. AV and TBA measure the secondary product of lipid oxidation. TBA measures the malonaldehyde produced during lipid oxidation. It can be assessed that if the PV value is 10-20 mg oxygen/kg or TBA is above 1-2 mg of malonaldehyde per kg of the sample, then the fish will in all probability smells and taste rancid. During prolonged storage of fish, PV, AV, and TBA values may increase reaching a peak and decline.

Spoilage of fish:

The process of quality deterioration or change in fish or fish product that renders it less acceptable, unacceptable, or unsafe for human consumption is known as spoilage. Spoilage can be

1. Microbial
2. Physical
3. Chemical

The most commonly used method for the quality evaluation of raw fish is sensory evaluation. Although the method is simple and rapid, the main disadvantage is the lack of objectivity. During spoilage, a number of chemical reactions are taking place in the fish muscle. Various

compounds are formed during these reactions, which are quantitatively determined, correlated with sensory characteristics, and used as spoilage indices.

Spoilage indices:

During spoilage, various compounds are produced in the fish muscle by autolytic enzymes, putrefactive microorganisms, or chemical reactions and gradually get accumulated in the flesh. Hence the quantitative determination of these compounds will provide a measure of the spoilage process. The spoilage indices for fish and shellfish are as follows:

1. Volatile bases
2. Nucleotides
3. Lipid oxidation products

Total Volatile Bases:

Volatile bases are produced by spoilage bacteria in fish. They are basic nitrogenous compounds such as ammonia, trimethylamine (TMA), Trimethylamine oxide (TMAO), and Dimethylamine *etc*., The most commonly used index of quality for the freshness of fish is the Total Volatile Base Nitrogen value (TVBN) along with Trimethylamine. Fish with a TVBN value of 20mg/100g is considered very fresh. The limit of acceptability of TVBN is 35-40 mg/100g beyond which the fish is considered as spoiled.

Trimethylamine (TMA):

Trimethylamine is the specific index used for assessing the freshness of marine fish. In most cases, the TMA concentration is extremely low, normally under 1mg N/100g. Studies indicate that in a few bivalves, the TMA content is about 20mg N/100g. In elasmobranchs and marine teleosts, the viscera, especially the spleen, liver, and kidney contain the most TMA and the muscle the least. The midgut gland has the highest level of TMA in squid.

TMA is derived from TMAO which is critical for osmoregulation in marine fish. Two types of enzymes are considered to be responsible for the reduction of TMAO to TMA and to DMA and formaldehyde (FA)- endogenous enzymes in fish, and exogenous enzymes produced by spoilage bacteria. The strains of bacteria capable of reducing TMAO to TMA have been found in most species of the Enterobacteriaceae including *Escherichia coli, Achromobacter, Micrococcus, Flavobacterium*, nonfluorescent *Pseudomonas, Clostridium, Alcaligenes,* and *Bacillus* spp. TMAO is reduced by bacterial enzymes to TMA while the endogenous enzymes reduce TMAO to DMA and then to FA. During frozen storage, the production of DMA is greater than that of TMA. Hence DMA can be used as an index of enzymatic deterioration during frozen storage and TMA as an index of pr-freezing quality. The

formation of DMA is accompanied by the equimolar formation of formaldehyde (FA), which can cause the denaturation of myofibrillar protein in fish flesh.

A level of 10-15 mg TMA-N/100g muscle is considered as the limit of acceptability. This level increases with storage time during iced storage hence TMA can be used as a good index of spoilage.

Ammonia:

Bacterial spoilage of fish generates small amounts of ammonia from the free amino acids. The ammonia content can be used as an indication of the extent of spoilage. A greater amount of ammonia is produced during the spoilage of elasmobranchs due to the high content of urea in their flesh. Shellfish can also produce a large amount of ammonia than marine fishes at the early stages of spoilage.

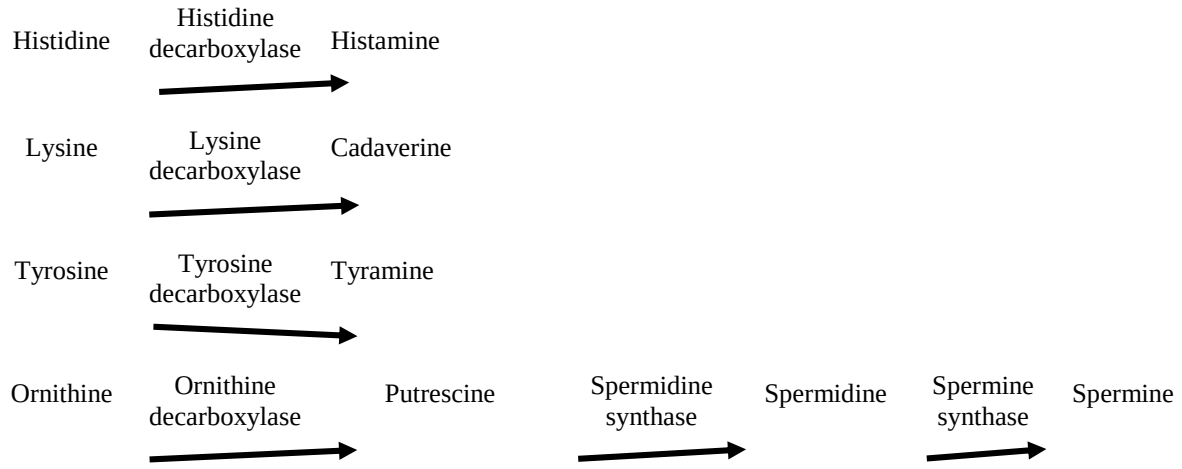
Biogenic amines:

Biogenic amines are non-volatile compounds, found at very low levels in fresh fish. Important biogenic amines are histamine, cadaverine, putrescine, tyramine, tryptamine, spermine and spermidine. Histamine is known to be the causative factor of scombroid poisoning/histamine poisoning in histamine-forming fishes such as mackerel, tuna, sardine, bonito, herring, anchovy *etc.,* Food Safety and Standards Authority of India has identified the following family of fishes as histamine forming fish species.

1. Carangidae – 30 species of fishes including jacks, scads, pompanos, queen fishes, kingfishes, and trevallies
2. Chanidae (Milkfish)
3. Clupeidae – 33 species of fishes including Sardine and Shad
4. Coryphaenidae (Mahi Mahi/Dolphin fish)
5. Engraulidae – 9 species of anchovy
6. Istiophoridae – 9 species of Marlin/Sailfish
7. Mugilidae (Mullet)
8. Pristigasteridae – 2 species of Ilisha/Pellona
9. Scombridae – 32 species of fishes including Mackerel, Tuna, Bonito, and Seer fish
10. Xiphiidae (Swordfish)

These fishes are found to be having high free histidine content which gets converted into histamine during spoilage. The biogenic amines formed during the spoilage of fish are found to be thermally stable and thus can be used as an indicator of poor quality of raw material in preserved/processed fishery products. Cadaverine and putrescine are found to be potentiators

of histamine. The direct precursors of histamine, cadaverine, and putrescine are histidine, lysine, and ornithine respectively. Putrescine is also an intermediate of a metabolic pathway that leads to the formation of spermidine and spermine.



Although biogenic amines have been associated with fish spoilage, the legal limit has been established for histamine only. As per Food Safety and Standards Regulation (FSSR, 2011), the maximum permissible level of histamine content in fish and fishery products is 200mg/Kg. Fishes with histamine content up to 20mg/kg are considered to be safe for consumption, 20-100mg/Kg is probably safe while ≥100mg/kg is toxic and unsafe for consumption.

Studies also indicated that cadaverine and putrescine can also be used as freshness indices for fish and shellfish respectively. Fish and fishery products containing cadaverine below 15mg/100g are considered as good for consumption, 15-20mg/100g indicates potential decomposition, and over 20mg/100g advanced decomposition. The quality Index (QI) and Biogenic Amine Index (Bai) are also used to indicate the freshness of fish.

$$QI = \frac{Histamine + Putrescine\ + Cadaverine}{[1 + (Spermidine + Spermine)]}$$

$$BAI = (Histamine + Putrescine + Cadaverine + Tyramine)$$

Indole: Indole is a spoilage indicator in shrimp and crab. Indole (2,3-benzopyrene) is a degradation product of tryptophan. Indole is highly volatile and soluble in different solvents such as hot water, alcohol, ether, and benzene. Shrimp with indole content <25mg/100g is organoleptically acceptable.

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Chapter 3

Chemical Contaminants in Fish and Fishery Products

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Introduction:

Global population is depending upon seafood as a healthy diet choice because of its richness in high value proteins, health beneficial vitamins, minerals and poly unsaturated fatty acids. Fish is also a primary protein source in most parts of the world. Even though fish supplies many health benefits, seafood can be compromised by different chemical contaminants which are harmful to consumers. Fishes are harvested from waters that are contaminated by varying amounts of industrial chemicals, heavy metals, pesticides and antibiotics. These contaminants may accumulate in fish at levels that can cause human health problems (e.g. carcinogenic and mutagenic effects). Food can become contaminated at any point during production, distribution and preparation. Everyone along the production chain, from producer to consumer, has a role to ensure the safety of seafood.

The number of chemical contaminants is increasing day by day, hence threats associated with chemical contamination of seafood is also increasing. Environmental contaminants mainly include ubiquitous pollutants such as heavy metals and dioxins. Even though they are naturally present in the environment their level can be increased due to anthropogenic influences. Contaminants can also come as toxins produced by fungi (Eg. aflatoxins) and algae (Eg. ciguatoxin). The different chemical contaminants in seafood can also include food additives that are intentionally added like preservatives, colour retention agents etc. The contaminants can also generate during processing or cooking which include acrylamide and heterocyclic amines. Residue of agricultural chemicals resulting from previous application of pesticides, and veterinary drugs during production and storage of food crops and animals, have been considered as human health hazards. But these types of contaminants have a great potential in control by proper conditions of usage and their presence. Also some natural components of food can also act as contaminant like allergic substances and phyto haemagglutinin.

Basically the chemical contaminants are classified into three main groups such as:

(i) **Naturally occurring** – allergens, Mycotoxins, Scomberotoxin (Histamine), Ciguatera poison, Puffer fish poison, Shellfish toxins (PSP, DSP, NSP, ASP)

(ii) **Unintentionally or incidentally added chemicals** – Pesticides, Fungicides, Fertilizers, Toxic compounds, Toxic metals

(iii) **Intentionally added chemicals and food additives** - Food preservatives, Food additives, Vitamins, Minerals, Antibiotics used in aquaculture, Sulfites used in shrimp to prevent melanosis, Nitrites as preservatives, Colouring agents, Detergents

Biotoxins

Marine biotoxins are responsible for many seafood borne diseases. It includes both shellfish toxins and ichthyotoxins (fish toxins). Shellfish toxins include Paralytic shellfish toxins, Diarrhetic shellfish toxins, Azaspriacid shellfish toxins, Neurotoxic shellfish toxin and Amnesic shellfish toxins. Ichthyotoxins include Ciguatera toxin and Tetradotoxin. Fish poisoning is caused by consuming fish containing poisonous tissues and shellfish poisoning results from ingestion of shellfish that have accumulated toxins from the plankton they have consumed.

(i)Tetradotoxin (Puffer fish poison): It is the most lethal of all fish poisons. Toxin production is due to the activity of symbiotic bacteria. Toxin will be accumulated in liver, ovaries and intestine as a defence mechanism. But the muscle is free of toxin. It is also called as Tetradon poisoning or Fugu poisoning. It is 275 times more toxic than cyanide. On an average a dose of 1-2mg of purified tetrodotoxin can be lethal to humans.

(ii) Ciguatera - Ciguatera is a clinical syndrome caused by eating the flesh of toxic fish caught in tropical reef and island waters. Most common fish poisoning and the fish becomes toxic due to feeding of toxic algae – dinoflagellates, *Gambeirdiscus toxicus*. Red snapper *(Lutjanus bohar),* Grouper *(Variola louti) and* Moray eel are recorded as ciguateric. More than 400 species have been implicated in ciguatera poisoning.

(iii)Paralytic shell fish poisoning (PSP) –This is associated with dinoflagellate blooms (*Alexandrium catenella, Gonyaulax tamerensis*). Heat stable saxitoxin will be accumulated inmussels, clams, oysters, scallops etc. grown in algal bloom areas. Greater number of human deaths is reported due to consumption of contaminated shellfish. The current regulatory level for fresh bivalve molluscs in most countries is 80 μg/100 g.

(iv)Diarrhetic shellfish poisoning (DSP) - Dinoflagellate *Dinophysis forti* is the algae which producesokadoic acid, the causative of DSP. Primary symptom is acute diarrhoea. Regulatory level in fresh bivalve molluscs in most countries is 0-60 μg /100 g.

Mouse bioassay and analysis by HPLC are the important methods for monitoring biotoxins. Reliable sampling plans are required for effective monitoring.

Heavy metals

Heavy metals are toxic metals and above a normal level can affect the quality, safety and marketability of seafood. They are "Cumulative poisons" which can irreversibly accumulate in the body. They have atomic weight higher than 40.04 and specific density > 5g/ cm. The main threats are Arsenic, Cadmium, Mercury and Lead. These metals have no beneficial effects in human and they have no homeostasis mechanism. These contaminants are highly depend upon geographic location, species and fish size, feeding pattern, solubility of chemical and their persistence in the environment.

Lead is mostly deposited in bones and not in soft tissues. But, from food safety point of view lead accumulation in edible parts is important. Compared to fish lead content is higher in shellfishes as it is getting accumulated in hepatopancreas. The organic form of lead, tetra alkyl lead is mostly found in fish. In fishes Cd is mostly deposited in kidney and liver and in muscles the level is quite low. In invertebrates like Cephalopods it can go as high as 30 ppm in digestive glands. Hence the digestive gland must be removed immediately after catch. Both Cd and Pb are carcinogenic in nature. Mercury is one of the most toxic heavy metal in the environment. Among metal contaminants methyl mercury ha s elicited the most concern among consumers. It is toxic to the nervous system especially the developing brain. Arsenic is a widely distributed metalloid and major contaminant in case of ground water. IARC has classified inorganic arsenic as a human carcinogen.

The most widely used techniques for detection and quantification of heavy metals are Atomic Absorption Spectrometry, Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS).

Histamine in fish

Though all types of biogenic amines can be formed in fish, the most toxic amine detected in fish is histamine. Histamine poisoning is the most common form of toxicity caused by ingestion of fish and is generally due to the ingestion of foods containing unusually high levels of histamine. The commonly implicated incidents of histamine poisoning are associated with the fish families Scombridae and Scomberesocidae. It is also known as Scombroid poisoning. Histamine is a powerful biologically active chemical present in the mast cells and basophils in larger amounts. Histamine poisoning is often manifested by a wide variety of symptoms. Major symptoms affecting the cutaneous system include rashes, urticaria, edema and localized

inflammation etc. gastrointestinal effects include nausea, vomiting, diarrhoea and abdominal cramps. Also include symptoms like hypotension, headache, palpitation, tingling and flushing. Severe suffocation and respiratory distress have been reported in severe cases of histamine poisoning. The onset of histamine poisoning can extend from 10 minutes to 1 hour following consumption of contaminated fish and can last from 12 hour to a few days. Histamine concentration required to produce poisoning varies with respect to the susceptibility of each individual. In case of susceptible individuals concentration between 5 and 10 mg/100g can cause symptoms. Many foods contain small amounts of histamine which can be tolerated easily.

As per USFDA guideline the toxicity and defect action level established are 50 mg/100g and 5 mg/100g respectively. According to EU regulation No 2073/2005 mean value all samples (nine) must not exceed 10 mg/100g, two samples may be > 10 mg/100g but < 20 mg/100g and no sample may exceed 20 mg/ 100g. According to USFDA guideline for the control of histamine production a core temperature of 4.4 ºC or less should be achieved and maintained throughout handling, processing and distribution of susceptible species.

A wide variety of procedure for the determination of histamine and biogenic amines is available. Include both semi quantitative and quantitative methods. Methods based on colorimetry, fluorometry and enzyme-linked immunosorbent assay (ELISA) are available. Mostly biogenic amines including histamine is analysed by High Performance Liquid Chromatography (HPLC) methods with pre and post column derivatisation and UV–visible or fluorescence detection. LC with tandem mass spectrometry (MS/MS) can also be a useful approach for an unequivocal confirmation of the studied analytes.

Antibiotics

Illegal use of antibiotics for veterinary purposes has become a matter of public concern. Antibiotics are used in aquaculture as prophylactics, as growth promoters and for treatment of diseases. They are usually administered in feeds and most commercial shrimp feeds contain antibiotics.The feeding of antibiotics as growth promoters is associated with decrease in animal gut mass, increased intestinal absorption of nutrients and energy sparing. But inappropriate and frequently abusive, use of antibiotics can affect human health. The two major concerns are the presence of antimicrobial residues in edible tissues and the emergence of antimicrobial resistance, which represents a huge threat to public health worldwide.

The greatest potential risk to public health associated with antimicrobial use in aquaculture is the development of a reservoir of transferable resistance genes in bacteria of

aquatic environments. The antibiotics lose their efficacy over time because of the emergence and dissemination of resistance among bacterial pathogens.

EU implemented "zero tolerance policy" regarding antibiotic residue. Using LCMSMS method EU laboratories are equipped to detect traces of prohibited carcinogenic antibiotics like chloramphenicol up to 0.3 ppb and nitrofuran up to 1 ppb levels. Many of the antibiotics are listed as prohibited substance in fish and fishery products. In India the tolerance limit has been set only for the following antibiotics

Antibiotic	**MRL (ppm)**
Tetracycline	0.1
Oxytetracycline	0.1
Trimethoprim	0.05
Oxolinic Acid	0.3

The monitoring of antimicrobial residues in fish tissues requires sensitive and selective analytical methodologies to verify the accomplishment of the legal framework and reach the desirable high standards of quality and food safety. The methods can be microbiological, immunochemical or physico chemical. European council directive 96/23/EC, 1996 gives direction on measures of monitoring residues in live and animal products. It specifies spectrometric detection, GC, HPLC, ELISA and LC-MS/MS methods.

Pesticides

Pesticides are substances used for preventing, destroying or controlling any pest. The major chemical types of pesticides include (i) Organochlorine pesticides – mostly banned because of its lipophilic nature. Have properties of bioaccumulation and high persistence (Eg: DDT and its derivatives, BHC, Endosulfan, aldrin, dieldrin etc). (ii) Carbamates – widely used insecticides (Eg: carbaryl, carbofuran, carbosulfan). (iii) Organophosphates – have rapid aciton at lower concentration, easy biodegradable in nature (Eg: malathon, Moncrotophos). (iv) Pyrethroids – have low mammalian toxicity and knock down effect against insects (Eg: Deltamethrin, Cypermethrin, Cyhalothrin, Fenvalerate etc.). Pesticide contamination in fish mainly comes though agricultural runoff and municipal sewage effluent.

Persistent organic pollutants (POPs) – they are organic chemicals that remain intact in the environment for long periods, become widely distributed, bio accumulate in food chain and are toxic to humans, wild life and environment. The POPs to which seafood consumers are most likely exposed are dioxins and PCBs. The Stockhome convention on POPs initially

identified twelve POPs, called as 'dirty dozen' include 9 pesticides, 2 industrial chemicals and 1 un intentional by product. They are aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, hexachlorobenzene, mirex, toxaphene, polychlorinated biphenyls (PCBs), dioxins and furans. Later nine new chemicals were again added to Stockhome convention.

The chromatographic techniques mainly Gas chromatography (GC), Gas chromatography-tandem mass spectrometry (GC-MS/MS) and Liquid chromatography-tandem mass spectrometry (LC-MS/MS) are used for the analysis of pesticide residues.

Food additives

Food additives means substances that normally are not used independently as food or its ingredient and which, after being added to the food during its production, processing packaging, transportation or storage, remain included in the food, even in changed state. In simpler terms, food additives are the substances which are added to food by the manufacturers to facilitate processing or to improve appearance, texture, flavour and keeping quality. Functions of food additives are

- To maintain product consistency – Eg: emulsifiers, stabilizers, thickners etc
- To improve nutritional quality – Eg: vitamins, minerals
- To improve product safety and quality – Eg: preservatives, antioxidants
- To aid in process or preparations – Eg: leavening agents
- To enhance sensory characteristics of the product

Classification of food additives

Food additives are classified based on their function in food, i.e. the purpose for which the additive has been incorporated in the food.

- antioxidants
- preservatives
- food colours
- food flavours
- emulsifiers and stabilizers
- anti-caking agents
- sequestrants
- acid, bases and buffers
- anti-foaming agents
- sweeteners
- enzymes, and leavening agents.

Chapter 4

Heavy Metal Residues in Fish and Fishery Products

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Heavy metals are toxic metals and above a normal level can affect the quality, safety and marketability of seafood. They have atomic weight higher than 40.04U and specific density > 5g/cm^3. Heavy metal contamination in fish and other aquatic organisms are highly depending upon geographic location, species and fish size, feeding pattern, solubility of chemical and their persistence in the environment. The major toxic heavy metals causing significant importance in seafood safety are Arsenic (As), Cadmium (Cd), Mercury (Hg) and Lead (Pb).

Compared to fish lead content is higher in shellfish as it is getting accumulated in hepatopancreas. The organic form of lead, tetra alkyl lead is mostly found in fish. In fishes Cd is mostly deposited in kidney and liver. In invertebrates like Cephalopods, it can go as high as 30 ppm in digestive glands. Hence the digestive gland must be removed immediately after catch. Both Cd and Pb are carcinogenic in nature. Mercury is one of the most toxic heavy metals in the environment. Among metal contaminants methyl mercury has elicited the most concern among consumers, affecting the nervous system. Arsenic is a widely distributed metalloid and a major contaminant in case of ground water. International Agency for Research on Cancer (IARC) has classified inorganic arsenic as a human carcinogen.

Being inhabitants of the aquatic ecosystem, fish and other aquatic species (molluscs, crustaceans, etc) carry the natural burden of heavy metal concentration. Heavy metals in fish and other aquatic organisms come from both natural and anthropogenic sources. The presence of toxic heavy metals such as lead, cadmium, mercury, arsenic, nickel, and chromium is of significant importance in seafood safety. Due to coastal pollution, in some areas of the Indian coast the enrichment factor for metals is very high (>100). In the aquatic environment, cadmium is also extensively distributed and bioaccumulation of cadmium by aquatic organisms is a well-recognized fact. The cephalopods (Squid, Cuttlefish, and Octopus) naturally bio-accumulate cadmium to toxic levels.

Similarly, predatory finfishes like Tuna, Marlin, Swordfish, and Barracuda, which contribute significantly to India's fish production are associated with high mercury levels. Mercury is present in fish primarily in its organic form as methyl mercury and accumulates with age.

Methylmercury accumulates rapidly but depurates very slowly. Because of this reason most mercury in fish muscle is present as methylmercury.

Although more than 90% of the mercury in fish is found as methylmercury, the contents of methylmercury can vary considerably between species. Predatory species that are at the top of the food chain and have long life span accumulate higher levels of methylmercury. Methyl mercury is known to cross the blood-brain barrier and placenta and cause irreversible prenatal and post-natal damage in the ingested population. Tuna and swordfish are found to be the main source of high methyl mercury (MeHg) exposure, followed by cod, haddock, and octopus.

Although Codex prescribes a limit for methyl mercury, many country regulations are based on total mercury content. Estimation of methyl mercury requires the use of cost-prohibitive hyphenated equipments like HPLC-ICP-MS or IC-ICP-MS. Similarly, high Arsenic content is reported in seafood, but major chemical forms are organic (arsenobetaine and arsenosugars), which are non-toxic.

Determination of heavy metals in seafood

Principle: Plasma is a stream of highly ionized gas containing an equal number of electrons and positive ions. Plasma is electrically conductive. It is affected by a magnetic field. When plasma energy is given to an analysis sample from outside, the component elements (atoms) are excited. When the excited atoms return to a low energy position, emission rays (spectrum rays) are released and the emission rays that correspond to the photon wavelength are measured. The element type is determined based on the position of the photon rays and the content of each element is determined based on the intensity of the rays.

To generate plasma, first argon gas is supplied to the torch coil, and high-frequency electric current is supplied to the work coil at the tip of the torch tube. Using the electromagnetic field created in the torch tube by the high-frequency current, argon gas is ionized and plasma is generated. This plasma has high electron density and temperature (10000K) and this energy is used in the excitation-emission of the sample. Solution samples are introduced into the plasma in an atomized state through the narrow tube in the center of the torch tube. The steps leading to the emission are desolvation, vaporization, atomization, and ionization.

Sample digestion

The sample should be homogenous, representative of bulk, free of suspended particles, and free-flowing. Samples are digested in a microwave digestion unit. Take 0.25 to 0.5 g of the sample to the pre-cleaned digestion vessel. Add 8 ml nitric acid and slowly add 2 ml H_2O_2 to it. Keep it for 10 minutes. Close the vessel and keep it in a microwave digestion chamber for

digestion. After digestion the samples are made up to 100 ml. digested sample is introduced to Inductively Coupled Plasma - optical emission spectrometry (ICP-OES) for analysis.

Chapter 5

Fundamentals of Bacteriology

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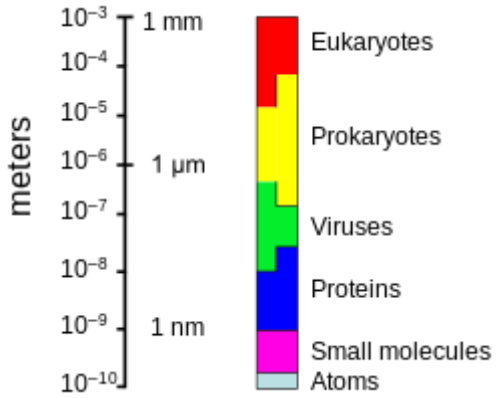
A microorganism, or microbe, is an organism of microscopic size, which may exist in its single-celled form or as a colony of cells. Technically a microorganism or microbe is an organism that is microscopic. The scientific study of microorganisms began with their observation under the microscope in the 1670s by Anton van Leeuwenhoek. The microorganisms are classified into Bacteria, Fungi, Archaea, Protists, Microscopic plants (green algae), Microscopic animals (plankton) and Virus. Microorganisms can be found almost anywhere on Earth. Bacteria and archaea are almost always microscopic, while a number of eukaryotes are also microscopic, including most protists, some fungi, as well as some micro-animals and plants. Bacteria like archaea are prokaryotic - unicellular, and having no cell nucleus or other membrane-bound organelle.

Bacteria function and reproduce as individual cells, but they can often aggregate in multicellular colonies. Some species such as myxobacteria can aggregate into complex swarming structures, operating as multicellular groups as part of their life cycle, or form clusters in bacterial colonies such as *E. coli*. Their genome is usually a circular bacterial chromosome – a single loop of DNA, although they can also harbor small pieces of DNA called plasmids. These plasmids can be transferred between cells through bacterial conjugation. Bacteria have an enclosing cell wall, which provides strength and rigidity to their cells. In general, bacteria are between 0.2 and 2.0 um - the average size of most bacteria. Research studies have shown their size to play an important role in survival over time. Due to their small size, bacteria are able to exploit and thrive in various microenvironments. The small size of bacteria is also beneficial for parasitism and oligotrophy.



The following are the major categories of bacteria based on their shapes:

a) Cocci: Cocci bacteria appear spherical or oval in shape. For the most part, the shape is determined by the cell wall of the organism and therefore varies from one type of cocci bacteria to another. Cocci bacteria may exist as single cells or remain attached to each other. Attached Cocci bacteria include: **Diplococci** bacteria - Diplococci bacteria are the type of cocci bacteria that occur as a pair (two joined cells). Some examples of Diplococci bacteria include: *Streptococcus pneumonia, Moraxella catarrhalis, Enterococcus* spp*, Neisseria gonorrhoea*. While some of these cells may be truly round shaped, others may appear elongated (ovoid) or bean-shaped/kidney shaped. For instance, some Neisseria cells may appear round while others are bean-shaped when viewed under the microscope. **Tetrad bacteria** - Tetrad bacteria are arranged in groups of four cells. Following division, the cells remain attached and grow in this attachment. Common examples of Tetrad bacteria include: *Pediococcus, Tetragenococcus.*

Sarcinae sarcina/Bacteria - Sarcina bacteria occur in groups of 8 cells. Unlike tetrads that divide into two planes, Sarcinae is produced through the perpendicular plane division. Some of the characteristics associated with these bacteria include being strict anaerobes, Gram-positive bacteria and that measure between 1.5 and 3.0 um. Examples of Sarcinae bacteria include: *Sarcina aurantiaca, Sarcina lutea, Sarcina ventriculi*. **Streptococci Bacteria**- Streptococci bacteria are a type of bacteria that arrange in a chain form (resembling chains). A majority of these bacterial cells are also ovoid in shape and may form paired chains. As members of the family Streptococcaceae, this group of bacteria is characterized by being non-motile, Gram-positive organisms. Examples of Streptococcus bacteria include: *Streptococcus pyogenes, Streptococcus pneumonia, S. mutans*. **Staphylococci Bacteria**- Staphylococci Bacteria are a type of bacteria that form grape-like clusters. This type of arrangement is the result of division that occurs in two planes. Two of the main characteristics of these organisms

are that they are immobile, Gram-positive bacteria. Examples of Staphylococci bacteria include: *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus aureus* *Staphylococcus capitis*.

b) Bacillus Bacteria (Rod-Shaped): Bacillus bacteria have the following traits: Are all rod-shaped, form endospores and are facultative anaerobes. bacillus bacteria are also arranged differently. While some exist as single, unattached cells (e.g. *Salmonella enterica* subsp, *Bacillus cereus*, and *Salmonella choleraesuis*), others are attached. The following are the different types of bacillus arrangements: *Diplobacilli bacteria* - Like Diplococci bacteria, Diplobacilli occur in pairs. Following cell division, the two cells do not separate and continue existing as a pair. Examples of Diplobacilli bacteria include: *Coxiella burnetii*, *Klebsiella rhinoscleromatis*, *Moraxella bovis*. **Coccibacilli bacteria** - Compared to other bacilli, Coccibacilli bacteria are shorter in length and thus appear stumpy. Examples of Coccibacilli include: *Chlamydia trachomatis*, *Haemophilus influenza*. Unlike cocci and bacilli bacteria, some types of bacteria appear curved when viewed under the microscope. However, they vary in shape making it possible to differentiate them from each other. These include: Vibrio bacteria - Generally, vibrio bacteria are comma-shaped and thus not fully twisted (curved rods). Examples of Vibrio bacteria include: *Vibrio mytili*, *Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Vibrio cholerae*. **Spirochete** - Spirochetes are characterized by a helical shape. Spirochetes are also flexible and have been shown to produce mycelium. The movement involves the use of axial filaments, which is one of the distinguishing features between the bacteria and other types of bacteria. Examples of Spirochetes include: Leptospira, Spirochaeta, Treponema. **Spirilla** bacteria - Like Spirochetes, Spirilla bacteria possess a helical shape. However, they are more rigid and have the typical flagella found in other types of bacteria. Some examples of Spirilla bacteria include: *Aquaspirillum*, *Campylobacter jejuni*, *Spirillum winogradskyi*.

In microbiology and bacteriology, Gram stain or Gram staining, also called Gram's method, is a method of staining used to classify bacterial species into two large groups: gram-positive bacteria and gram-negative bacteria. The name comes from the Danish bacteriologist Hans Christian Gram, who developed the technique in 1884. Gram staining differentiates bacteria by the chemical and physical properties of their cell walls. Gram-positive cells have a thick layer of peptidoglycan in the cell wall that retains the primary stain, crystal violet. Gram-negative cells have a thinner peptidoglycan layer that allows the crystal violet to wash out on addition of ethanol. They are stained pink or red by the counterstain, commonly safranin or fuchsine.

Lugol's iodine solution is always added after addition of crystal violet to strengthen the bonds of the stain with the cell membrane. Gram staining is almost always the first step in the preliminary identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique. Acid-fast staining is the differential staining techniques which was first developed by Ziehl and later on modified by Neelsen. So this method is also called Ziehl-Neelsen staining techniques. Neelsen in 1883 used Ziehl's carbol-fuchsin and heat then decolorized with an acid alcohol, and counter stained with methylene blue. Thus Ziehl-Neelsen staining techniques was developed. The main aim of this staining is to differentiate bacteria into acid fast group and non-acid fast groups. This method is used for those microorganisms which are not staining by simple or Gram staining method, particularly the member of genus Mycobacterium, are resistant and can only be visualized by acid-fast staining.

Growth Curve

In a closed system with enough nutrients, a bacteria shows a predictable growth pattern that is the bacterial growth curve. It consists of four different phases. Read on to learn about the phases in detail. Phases of the Bacterial Growth Curve: Upon inoculation into a new nutrient medium, the bacteria shows four distinct phases of growth. Let us dive into each of the phases in detail.

Lag Phase: The bacteria upon introduction into the nutrient medium take some time to adapt to the new environment. In this phase, the bacteria does not reproduce but prepares itself for reproduction. The cells are active metabolically and keep increasing in size. The cells synthesise RNA, growth factors and other molecules required for cell division.

Log Phase: Soon after the lag phase, i.e., the preparation phase, the bacterial cells enter the log phase. The log phase is also known as the exponential phase. This phase is marked by the doubling of the bacterial cells. The cell number increases in a logarithmic fashion such that the cell constituent is maintained. The log phase continues until there is depletion of nutrients in the setup. The stage also comes to a stop if toxic substances start to accumulate, resulting in a slower growth rate. The cells are the healthiest at this stage and researchers prefer to use bacteria from this stage for their experimental processes. Plotting this phase on the bacterial growth curve gives a straight line. Upon calculation of the slope of this line, the specific growth rate of the organism is obtained. It is the measure of divisions per cell per unit of time.

Stationary Phase: In the stationary phase, the rate of growth of the cells becomes equal to its rate of death. The rate of growth of the bacterial cells is limited by the accumulation of toxic

compounds and also depletion of nutrients in the media. The cell population remains constant at this stage. Plotting this phase on the graph gives a smooth horizontal linear line.

Death Phase: This is the last phase of the bacterial growth. At this stage, the rate of death is greater than the rate of formation of new cells. Lack of nutrients, physical conditions or other injuries to the cell leads to death of the cells.

Physical factors that affect microbial growth

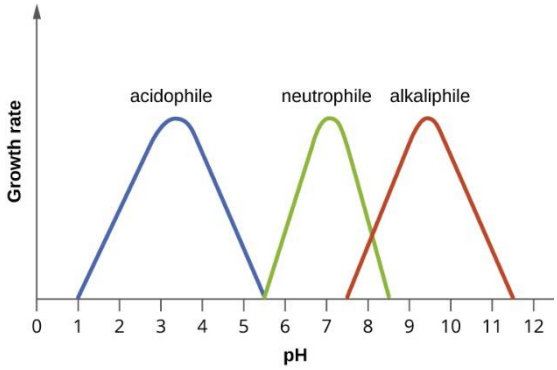
a) Temperature: Generally,an increase in temperature will increase enzyme activity. But if temperatures get too high, enzyme activity will diminish and the protein (the enzyme) will denature. On the other hand, lowering temperature will decrease enzyme activity. At freezing temperatures enzyme activity can stop. Repeated cycles of freezing and thawing can denature proteins. In addition, freezing causes water to expand and also forms ice crystals, hence cells begin to rupture. Every bacterial species has specific growth temperature requirements which is largely determined by the temperature requirements of its enzymes. PSYCHROPHILES grow best between -5^{o}C and 20^{o}C, MESOPHILES grow best between 20^{o}C and 45^{o}C and THERMOPHILES grow best at temperatures above 45^{o}C. THERMODURIC organisms can survive high temperatures but don't grow well at such temperatures. Organisms which form endospores would be considered thermoduric. Some organisms have exotic temperature requirements. *Thermus aquaticus* is a bright orange gram negative rod isolated from hot water and steam vents at Yellowstone Park. This organism grows best at temperatures between 70-75^{o}C (158-167^{o}F). Some of its unique enzymes are in demand for molecular biological and industrial applications.

b) **Oxygen**: Microbes display a great diversity in their ability to use and to tolerate oxygen. In part this is because of the paradoxical nature of oxygen which can be both toxic and essential to life. OBLIGATE AEROBES rely on aerobic respiration for ATP and they therefore use oxygen as the terminal electron acceptor in the electron transport chain. Pseudomonas is an example of this group of organisms. MICROAEROPHILES require O_2 for growth but they are damaged by normal atmospheric levels of oxygen and they don't have efficient ways to neutralize the toxic forms of oxygen such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The Streptococci are examples of this group. OBLIGATE ANAEROBES will die in the presence of oxygen because they lack enzymes like superoxide dismutase and catalase. Organisms like Clostridium, metabolize through fermentation and / or anaerobic respiration. AEROTOLERANT organisms like Lactobacillus ferment and therefore do not use oxygen, however they do tolerate it. FACULTATIVE ANAEROBES are the most adaptable. They are

capable of both fermentation and aerobic respiration. *Escherichia coli* is an example of this class of organisms. ANAEROBIC PATHOGENS: *Clostridium tetani* - agent of tetanus, puncture wounds, produces a toxin which enters the spinal column and blocks the inhibitory spinal motor neurons. This produces generalized muscle spasms or spastic paralysis. *Clostridium botulinum* - this soil organism is the causative agent of botulism which typically occurs after eating home canned alkaline vegetables which were not heated enough during canning. The neurotoxin blocks transmission across neuromuscular junctions and this results in flaccid paralysis. *Clostridium perfringes* and *Clostridium sporogenes* - these organisms are associated with invasive infections known as GAS GANGRENE. *Clostridium difficile* - the causative agent of pseudomembranous colitis, a side effect of antibiotic treatment which eliminates the normal flora. MICROAEROPHILES: These organisms are all catalase negative, therefore the catalase test is useful in identification. They also have distinctive colonial morphology on blood agar which is differential for them. It is important to note if the colonies are alpha, beta, or gamma hemolytic. Group A Streptococcus - *Streptococcus pyogenes,* This beta hemolytic organism is also bacitracin sensitive. It is the cause of strep throat, rheumatic fever, glomerulonephritis and scarlet fever. Group D Streptococcus - Enterococcus - *Streptococcus faecalis,* This organism is a normal inhabitant of the large intestine. It is also a frequent cause of bladder infections. *Streptococcus pneumonia,* This organism is a normal inhabitant of the respiratory tract. It is a frequent cause of pneumonia in people who have been compromised by other illness.

Based on the nutritional requirements, bacteria are classified as follows:

Energy source:	light:	phototrophic
	chemical:	chemotrophic
Electron source:	inorganic compounds:	lithotrophic
	organic compounds:	organotrophic
Carbon source:	CO_2:	autotrophic
	organic:	heterotrophic



Based on pH bacterial requirements are classified as follows:

Most bacteria are neutrophiles, meaning they grow optimally at a pH within one or two pH units of the neutral pH of 7. Most familiar bacteria, like *Escherichia coli*, *Staphylococci*, and *Salmonella* spp. are neutrophiles and do not fare well in the acidic pH of the stomach. However, there are pathogenic strains of *E. coli*, *S. typhi*, and other species of intestinal

pathogens that are much more resistant to stomach acid. In comparison, fungi thrive at slightly acidic pH values of 5.0-6.0.Microorganisms that grow optimally at pH less than 5.55 are called acidophiles. Eg. *Lactobacillus* bacteria. Acidophilic microorganisms display a number of adaptations to survive in strong acidic environments. For example, proteins show increased negative surface charge that stabilizes them at low pH. Pumps actively eject H^+ ions out of the cells. At the other end of the spectrum are alkaliphiles, microorganisms that grow best at pH between 8.0 and 10.5. *Vibrio cholerae*, the pathogenic agent of cholera, grows best at the slightly basic pH of 8.0; it can survive pH values of 11.0.

Chapter 6

Incidences of Pathogens in Fish and Fishery Products

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Fish and fishery products consumption trend has increased in recent times because of globalization and healthy food awareness. Changes in life style of human has also contributed for pace in consumption. Foodborne illness (commonly known as food poisoning) is often caused by consuming food contaminated by bacteria and/or their toxins, parasites, viruses, chemicals, or other agents. Fish and fishery products may get contaminated with various pathogenic bacteria due to unhygienic handling practices which may results in food poisoning. Biological contaminants of food are harmful and hazardous substances of biological origin in the food that can cause foodborne illness when they are consumed.

Each year worldwide, unsafe food causes 600 million cases of foodborne diseases and 4,20,000 deaths. 30% of foodborne deaths occur among children under 5 years of age. WHO estimated that 33 million years of healthy lives are lost due to eating unsafe food globally each year, and this number is likely an underestimation.

Contributing to this underestimation is that many foodborne illnesses lack the severity, duration, and specific diagnosis required for definitive identification and intervention. Biological contaminants could be microorganisms, so small that only can be seen by a microscope such as bacteria and versus, or could be large such as some parasites. Bacteria can grow and multiply rapidly if food is not taken care for temperature. Bacterial and viral Pathogens are the primary food safety concern with regard to fish and fishery products. Some types of fish may also contain naturally occurring parasites. Poor handling practices, such as failure to prevent raw foods from coming in contact with cooked or ready-to eat foods (cross contamination), and lack of proper temperature control are significant factors that can lead to pathogen growth and foodborne illness. To prevent the outbreak of foodborne illnesses, it is crucial for food service professionals to understand all aspects of biological contaminants from how they grow and reproduce to how they contaminate food and infect humans. These hazards can come from raw materials or from food processing steps.

Bacterial Pathogens

Bacterial Pathogens are very common in fish and fishery products including viral pathogens and parasites. Bacterial Pathogens Pathogen contamination and growth is often an important

factor in food-borne illness. Pathogenic bacteria can cause illness in human, either by infection or intoxication. Food borne infections are caused by swallowing live pathogens that grow within the body, usually in the intestinal tract. Intoxication is a condition caused by swallowing preformed toxins means toxins produced by microorganisms in the food before it is eaten. Most of the pathogenic bacteria are not present in fish caught from off-shore waters, but contamination occurs during handling of the material. If the time and temperature conditions are favourable, these organisms get an opportunity to grow and multiply at a faster rate. Consumption of such fish is dangerous and it will lead to food poisoning.

Bacterial Pathogens:

1. *Aeromonas spp.*
2. *Bacillus cereus*
3. *Campylobacter jejuni*
4. *Clostridium botulinum*
5. *Clostridium perfringens*
6. *Pathogenic Escherichia coli*
7. *Listeria monocytogenes*
8. *Salmonella spp.*
9. *Shigella spp.*
10. *Pathogenic Staphylococcus aureus*
11. *Faecal Streptococci*
12. *Plesiomonas shigelloides*
13. *Pathogenic Vibrio spp.*
    a. *Vibrio cholerae*
    b. *Vibrio parahaemolyticus*
    c. *Vibrio vulnificus*
14. *Yersinia enterocolitica*

***Aeromonas* spp.**

The genera *Aeromonas* comprise Gram-negative, facultatively anaerobic, oxidasepositive, glucose-fermenting rod-shaped bacteria, generally motile. *Aeromonas* species viz. *A. hydrophila, A. sorbia* and *A. caviae* has been described as emerging food-borne pathogens. Besides gastroenteritis *A. hydrophila* may cause cholera like infections. *Aeromonas spp.* are natural members of aquatic environments and is commonly found in fish and fish products of

all aquatic environments. *A. hydrophila* is very resistant organism and it can survive in food items stored in cold for long period. Oysters have been implicated in food-borne disease.

Aeromonas associated diarrhoea has been reported from different parts of India. Some *Aeromonas* spp. are psychrotrophs and some others are enteropathogenic. Studies have shown that very high percentage of the isolates from fish and fishery products produced hemolysin (79.2%) and cytotoxin (91.7%). Psychrotrophic *Aeromonas* strains are able to grow at 4-5°C and produce toxin in oysters at 5°C. Combination of chilling, salting and/or acidification is effective means of preventing the growth of *Aeromonas.*

Bacillus cereus

Bacillus cereus is a facultatively anaerobic, catalase-positive, toxin-producing gram-positive bacterium found in soil, vegetation, and food. It commonly causes intestinal illnesses with nausea, vomiting, and diarrhea. However, it has been associated with serious infections in immuno-compromised hosts and can cause septicemia as well as endophthalmitis, which can lead to vision loss. Bacillus cereus is a well-known cause of food-borne illness, but infection with this organism is not commonly reported because of its usually mild symptoms. A fatal case due to liver failure Food poisoning caused by B. cereus may occur when foods are prepared and held without proper refrigeration for several hours before being served. B. cereus is an aerobic spore-forming bacterium. It is commonly found in soil, on vegetables, and in many raw and processed foods. Two types of illnesses have been attributed to B. cereus. The first is characterized by abdominal pain and diarrhea. It has an incubation period of 4-16 hours and symptoms that last for 12-24 hours. The second is characterized by an accute attack of nausea and vomiting. It has an incubation period of 1-5 hours. Diarrhea is not common with the second type of illness. Colonies of B. cereus have an irregular perimeter and are opaque on sheep blood agar. When grown on an egg yolk agar, a zone of opacification will be noted due to lecithinase production.

B. cereus is a common food contaminant. Effective control measures depend on Notes: destruction by a heat process and temperature control to prevent spore germination and multiplication of vegetative cells in cooked, ready-to-eat foods. Measures to reduce or eliminate the threat of food poisoning by B. cereus include: 1) Avoid preparing food too far in advance of planned service, 2) Avoid holding cooked foods at room temperature, 3) Use quick chill methods to cool foods below 45ºF (7.2ºC) within 4 hours of preparation; store in shallow pans/ small quantities with the food less than 4 inches deep; if food is especially thick (e.g.,

refried beans), store no more than 3 inches deep, 4) Hold/store hot foods above 140ºF (60ºC) until served, and 5) Reheat foods rapidly to 165ºF (74ºC) or above.

Campylobacter jejuni

They are very small, Gram-negative, microaerophilic, curved thin rods with corkscrew motility. C. jejuni is widely distributed in the intestinal tract of poultry, livestock, and warm-blooded domestic animals. It is a very common and important cause of diarrheal illness in humans. Symptoms include profuse diarrhea (sometimes bloody), abdominal pain (intensity and duration can be somewhat severe), headache, weakness, and fever. Many infections occur without symptoms. C. jejuni is transmitted through: contaminated foods, including raw clams, mussels and oysters; person-to-person contact; and contaminated water. Cross-contamination of foods by dirty food-contact surfaces, including cutting boards and hands, may be the most frequent route of transmission. Since the infective dose of C. jejuni is thought to be small, time/temperature abuse of food products is not necessary to result in this illness. *Campylobacter jejuni* is widely distributed in the intestinal tract of poultry, live-stock and warm-blooded domestic animals.

Contaminated food including raw clams, mussels and oysters, person to person contact, cross contamination of food by dirty food contact surface etc. Incubation period is 3-5 days. Profuse diarrhoea, abdominal pain, headache and fever and meningitis in neonates. Infective dose ranges from 500 to 10,000 cells. This organism survives refrigeration and freezing.

C. jejuni can be controlled by thoroughly cooking fish and fishery products and by stressing the importance of proper (and frequent) hand and equipment washing and sanitary food-handling practices.

Clostridium botulinum

Clostridium botulinum is a dangerous food poisoning organism and it produce a very deadly, exotoxin when grows in food. The food poisoning is known as 'botulism''. It is an anaerobic, Gram-positive, spore-forming rod. The spores are highly heat resistant. Eight different toxins i.e. A, B, C1, C2, D, E, F & G known to exist. Type- E is present in sea mud and is mostly involved in botulism food poisoning in fish and fishery products. Food poisoning is due to the ingestion of toxin.

C. botulinum is found throughout the environment and has been isolated from soil, water, vegetables, meats, dairy products, ocean sediments, the intestinal tracts of fish, and the gills and viscera of crabs and other shellfish. C. botulinum is a spore-forming bacteria that grows in the absence of air. These characteristics allow it to survive normal cooking temperatures and

to grow in a vacuum packaged and modified-atmosphere environment. C. botulinum produces a powerful neurotoxin that causes botulism. Growth is necessary for C. botulinum to produce toxin. Symptoms include diarrhea, vomiting, abdominal pain, nausea and weakness. These are followed by double, blurred vision and dilated, fixed pupils. In severe cases, paralysis of the muscles responsible for breathing can cause death. The type of C. botulinum Type E that is most common in fish and fishery products is of particular concern because it grows at temperatures as low as 38 F and produces little noticeable evidence of spoilage. C. botulinum Type A is the form of this bacteria that is most common in land-based products. It is a common contaminant on processing equipment. It will grow at temperatures no colder than 50 F and produces a putrid odor in products in which it grows. However, its spores are much more heat-resistant than the Type E form of the bacteria.

Because *C. botulinum* produces heat-resistant spores and requires the absence of oxygen for growth, botulism has been most commonly associated with improperly canned food (usually home canned). Semi-preserved fish and fishery products, including smoked, salted and fermented fish, have also been identified as causes of botulism.

C. botulinum can be controlled by inhibiting growth of the bacteria or by destroying it in fish and fishery products. Proper thermal processes for canned fish and fishery products destroy the bacteria. Heavy salting or drying to reduce the water activity below 0.93 and fermentation or acidification to below pH 4.6 are effective means of preventing C. botulinum growth. Maintaining proper storage temperatures alone is not considered an adequate control measure for C. botulinum Type E because of its ability to grow at low temperatures and because of the severity of the illness. Nonetheless, in many products, it is an important second barrier to growth.

Clostridium perfringens

C. perfringens is commonly found in soil, dust, and the intestinal tract of animals. It is a spore forming, anaerobic (oxygen-free growth conditions) bacterium. Food poisoning caused by C. perfringens may occur when foods are cooked and held without maintaining adequate heat or refrigeration before serving. The illness is a self-limiting gastroenteritis with an incubation period of 8-15 hours and a duration of 12-24 hours. The symptoms, which include intense abdominal cramps, gas, and diarrhea, have been attributed to a protein enterotoxin produced during sporulation of the organism in the intestine.

The presence of small numbers of C. perfringens is not uncommon in raw meats, poultry, dehydrated soups and sauces, raw vegetables, and spices. Because the spores of some strains

are resistant to temperatures as high as 100ºC for more than 1 hour, their presence in foods may be unavoidable. Furthermore, the oxygen level may be sufficiently reduced during cooking to permit growth of the clostridia. Spores that survive cooking may germinate and grow rapidly in foods that are inadequately refrigerated after cooking. Thus, when clinical and epidemiological evidence suggests that C. perfringens is the cause of a food poisoning outbreak, the presence of hundreds of thousands or more of these organisms per gram of food substantiates the diagnosis.

Control measures emphasize proper food preparation and storage techniques, especially temperature control. Control measures include: Rapid, uniform cooling of cooked foods of cooked foods to < 10ºC (50ºF) within 2-3 hours; Hot holding of cooked foods at or above 60ºC (140ºF); Reheating cooled or chilled foods to a minimum internal temperature of 75ºC (167ºF) immediately before serving; Not leaving foods at room temperature or thawing frozen foods at room temperature; Preventing cross-contamination of cooked foods with bacteria from raw foods by using separate food-contact surfaces for preparing raw and cooked foods items, or by thoroughly cleaning and sanitizing food contact surfaces after being used for raw products; Maintaining food preparation areas so that they are free of soil and dust; Cleaning and sanitizing meat slicers, meat-cutting equipment, food contact surfaces, and other equipment after use; and Using good personal hygiene methods, and thoroughly washing hands frequently when handling food products, especially after handling raw products and before handling cooked products.

Escherichia coli

E. coli are Gram-negative, rod-shaped, non-spore forming facultatively anaerobic bacteria. E. coli are naturally found in the intestinal tracts of all animals, including humans. Most forms of the bacteria are not pathogenic and serve useful functions in the intestine. Pathogenic strains of *E. coli* are transferred to fish and fishery products through sewage pollution of the coastal environment or by contamination after harvest. E. coli food infection causes abdominal cramping, water or bloody diarrhea, fever, nausea, and vomiting.

Generally this organism is harmless: Pathogenic strains of E.coli are considered to be harmful.

- Enterotoxigenic E.coli (ETEC) - Gastroenteritis
- Enteropathogenic E.coli (EPEC) - Infant diarrhoea
- Enteroinvasive E.coli (EIEC) - Bacillary dysentery
- Enterohemorrhagic E.coli (EHEC) - Newly added category

- Enteroadherent E.coli (EAEC) - Hemorrhagic colitis (E.coli 0157:H7)

E. coli can be prevented by heating fish and fishery products sufficiently to kill the bacteria, holding chilled fish and fishery products below 40^0F, preventing post cooking cross-contamination, and prohibiting people who are ill from working in food operations. The infective dose of E. coli is dependent upon the particular strain from only a few organisms to millions. For this reason, time/temperature abuse of food products may or may not be necessary to result in illness.

Listeria monocytogenes

It is generally accepted as a food borne pathogen. Outbreak of disease is very rare but considered to be very serious due to high rate of mortality. *L. monocytogenes* is widely distributed in nature. A variety of animals can serve as hosts for this organism. The bacterium is often associated with the intestinal tract of domestic animals, birds and humans. About 1% of human population is known to carry *L. monocytogenes.*

This organism is Gram-positive, micro-aerophilic, non-spore forming, motile rods. It can survive freezing and thawing; if the load is more than 5x104 /ml. in milk it can withstand pasteurization. *L. monocytogenes* grows in refrigerated temperatures (even 1° C) and it can survive both acidic and alkaline pH. This is the most heat resistant pathogenic bacteria among non-spore formers.

L. monocytogenes is widespread in nature and has been isolated from soil, vegetation, marine sediments and water. In the early 1900s, *L. monocytogenes* was recognized as a bacterium that caused illness in farm animals. More recently, it has been identified as the cause of listeriosis in humans. Most healthy individuals are either unaffected by *L. monocytogenes* or experience only mild flulike symptoms. Victims of severe listeriosis are usually immunocompromised. Those at highest risk include: cancer patients, individuals taking drugs that affect the body's immune system, alcoholics, pregnant women, persons with low stomach acidity and individuals with AIDS. Severe listeriosis can cause meningitis, abortions, septicemia and a number of other maladies, some of which may lead to death.

The greatest threat of listeriosis is from ready-to-eat products that do not require further cooking at home. *L. monocytogenes* in raw food that will be cooked before consumption is less of a concern to the food industry since the bacteria are killed during cooking. *L monocytogenes* has been isolated from raw fish, cooked crabs, raw and cooked shrimp, raw lobster, surimi and smoked fish. One of its most significant characteristics is its ability to grow at temperatures as low as 31ºF.

L. monocytogenes can be prevented by thoroughly cooking fish and fishery products and by preventing cross-contamination once the fish and fishery products is cooked. Since the infective dose of *L. monocytogenes* is thought to be small, time/temperature abuse of food products may not be necessary to result in illness.

Salmonella spp.

Salmonella are enteric organisms producing enteric fever and food borne gastroenteritis. More than 2500 serotypes of this organism are known to exist at present and more are added to the list every year. Food poisoning due to salmonella is known as "Salmonellosis" infants, elderly and the under nourished are more susceptible to the disease and in such individuals salmonellosis is known to occur even from one single cell of Salmonella.

Salmonella are non-spore forming, mostly motile (exception *S. pullorum* and *S. gallinarum*) facultative — anaerobic, Gram-negative rods.

Salmonella is naturally found in the intestinal tracts of mammals, birds, amphibians and reptiles but not in fish, crustaceans or mollusks. *Salmonella* is transferred to fish and fishery products through sewage pollution of the harvest environment or by contamination after harvest. Freshly caught marine fish are usually free from *Salmonella*. However, fish from polluted coastal waters are usually contaminated with this organism.

Salmonella food infection causes nausea, vomiting, abdominal cramps and fever. Outbreaks of *Salmonella* food infection have been associated with raw oysters, salmon, tuna salad, shrimp cocktail, stuffed sole and gefilte fish.

Salmonella can be prevented by: heating fish and fishery products sufficiently to kill the bacteria, holding chilled fish and fishery products below 40 F, preventing post-cooking cross-contamination and prohibiting people who are ill or are carriers of Salmonella from working in food operations. The infective dose of Salmonella is thought to be extremely variable, relatively high for healthy individuals and very low for at-risk individuals, such as the elderly or medically compromised. For this reason, illness could result even without time/temperature abuse, but abuse has been a contributing factor in many outbreaks.

Shigella spp.

The disease caused by *Shigella* is generally known as `shigellosis' which is not indigenous in foods, transmitted through food or water contaminated with human excreta. *Shigella* are Gram-negative, facultatively anaerobic, non-sporulating, non-motile, rod-shaped bacteria. They are the most difficult enteric pathogens to isolate. Man is the only known natural host for *Shigella.*

The organisms pass the acid barrier of the intestine, multiply in the gut and produce ulceration of large intestine followed by dysentery.

Four serological groups i.e. A, B, C, and D. The major species in shigella comprises *Shigella dysenteriae; S. flexneri; S. boydi* and *S. sonnei. S. dysenteriae* causes the most severe illness They survive longest when food holding temperatures are 25°C or lower.

Shigella is naturally found in the intestinal tract of humans. Shigella is transferred to fish and fishery products through sewage pollution of the coastal environment or by contamination after harvest. Shigella produces an illness called Shigellosis, which causes mild diarrhea, fever, abdominal cramps and severe fluid loss.

Hazards from Shigella can be prevented by eliminating human waste contamination of water supplies and by improved personal hygiene for people who are ill or are carriers of Shigella and work in food operations.

Staphylococcus aureus

Since 1930, it is known that contamination of food with coagulase — positive staphylococci could cause food poisoning, as the organism growing in food materials in considerable numbers, secretes exotoxin. Staphylococcal food poisoning is caused only by certain well defined strains of S. aureus; such strains are known as enterotoxigenic strains. Food-borne out breaks due to coagulase-negative strains of Staphylococci are seldom reported. S. aureus are known to produce 9 different types of enterotoxins designated as enterotoxin A, B, C1, C2, D, E, F, G and H. This is the most drought resistant pathogenic bacteria and they cannot compete with general bacterial flora.

Humans and animals are the primary reservoirs for *S. aureus*. *S. aureus* can be found in the nose and throat and on the hair and skin of 50 percent of healthy individuals. However, the bacteria can be found in air, dust, sewage and surfaces of food-processing equipment. *S. aureus* can produce a toxin if allowed to grow in food. The toxin is not destroyed by the cooking or canning processes. *S. aureus* has the ability to grow and produce toxins in food with very little available water (.85 aw, 10 percent salt), which would prevent the growth of other pathogens.

S. aureus food poisoning causes nausea, vomiting, abdominal cramping, watery or bloody diarrhea, and fever.

Hazards from S. aureus can be prevented by: minimizing time/temperature abuse of fish and fishery products, especially after cooking, and requiring that food handlers engage in proper hygiene.

Faecal *Streptococci*

Faecal streptococci are Gram-positive, facultative anaerobic, non-spore forming non-motile and catalase negative cocci. Faecal streptococci are comparatively resistant to many adverse conditions. About 30% reduction of faecal streptococci takes place during freezing at -40°C, during subsequent storage at -18°C not much of reduction in count takes place even after 2 years of storage.

Primary habitat and source of contamination are same as in the case of E. coli. One gram of faeces contains 10^6 to 1^{08} faecal streptococci, therefore their presence in food product is generally regarded as an indication of faecal contamination. Just like E. coli, faecal streptococci are absent in off-shore water but are present in considerable numbers in coastal waters. Unclean boat deck, utensils, water and ice are the major source of contamination.

Vibrio cholerae

It is the causative agent of cholera. The current definition of V *cholerae* consists of the classical (non-hemolytic) and El Tor (hemolytic) biovars. The El Tor *vibrios* are generally more infectious than the classical *V. choleraeserotypes* and it can survive longer in the environment. The only natural habitat of *V. cholerae* is man. V. cholerae is found in estuaries, bays, and brackish waters. It is naturally occurring and is not necessarily related to sewage contamination. V. cholerae tends to be more numerous in the environment during warmer months.

There are a number of types of V. cholerae, and these produce very different symptoms. One type, Vibrio cholerae 01, initially causes abdominal discomfort and mild diarrhea. As the illness progresses, the symptoms may include: watery diarrhea, abdominal cramps, vomiting and dehydration. Death can occur. Susceptibility to cholera is enhanced in people who have had gastric surgery, take antacids or have type O blood. Outbreaks of this type of cholera have been associated with oysters, crabs and shrimp from the Gulf of Mexico. V. cholerae 01 has also been recovered from Chesapeake Bay waters, although no illness has been reported from that area.

Another type of *V. cholerae*, non-01, causes diarrhea, abdominal cramps and fever. Nausea, vomiting and bloody diarrhea have also been reported. The severity of the symptoms is dependant, in part, upon the specific strain. In its most severe form, V. cholerae non-01 has resulted in septicemia (blood poisoning) in individuals with medical conditions that weaken their immune systems. The illness has been associated with consumption of raw oysters, but the bacterium has also been found in crabs.

Hazards from *V. cholerae* can be prevented by cooking fish and fishery products thoroughly and by preventing cross-contamination once the fish and fishery products is cooked.

Vibrio parahaemolyticus

V. parahaemolyticus is a marine pathogen present in marine and brackish-water. They are Gram-negative, rod shaped bacteria which are non-sporulating, halophilic, motile, and oxidase-positive. *V. parahaemolyticus* is naturally occurring in estuaries and other coastal areas throughout most of the world. In most areas, *V. parahaemolyticus* is more numerous in the environment during the warmer months and, as a result, most outbreaks occur during the summer.

The most commonly experienced symptoms of *V. parahaemolyticus* illness include: diarrhea, abdominal cramps, nausea, vomiting and headache. Fever and chills are less frequently reported. The illness has been associated with consuming contaminated crabs, oysters, shrimp and lobster.

Hazards from *V. parahaemolyticus* can be controlled by thoroughly cooking fish and fishery products and preventing cross-contamination after cooking. Control of time/temperature abuse is also an important preventative measure.

environments. It can cause food poisoning when it is consumed in large numbers (more than 10^5/g of Kanagawa-positive strains), along with food materials. This type of food poisoning is more in countries like Japan, where there is a habit of eating un-cooked fish and fishery products. In recent years, the incidence of *V. parahaemolyticus* infection has been increasing in many parts of the world, and this has been attributed to the emergence of a new clone of the 03:K6 serotype carrying only the *tdh* gene. The onset of symptoms is within 12 h of eating infected food.

Icing the material immediately after catch, washing with potable water and improvement of hygiene are considered as remedial measures.

Vibrio vulnificus

V. vulnificus is a naturally occurring marine bacterium. It is an emerging pathogen, phenotypically similar to *V. parahaemolyticus.* Mortality is up to 60%. It is the part of the normal bacterial flora of estuarine and marine waters.

V. vulnificus is Gram-negative, halophilic, lactose-positive, rod shaped bacteria. All strains are pathogenic; infection dose is not known! Infection is associated with the consumption of raw fish and fishery products particularly oysters.

Vibrio vulnificus requires salt for survival and is commonly isolated at salinities of 7 ppt to 16 ppt. It is primarily found in the Gulf of Mexico, but it has also been isolated from the Atlantic and Pacific oceans. The numbers of the bacterium in the environment are highest during the warmer months of April through October.

The most common symptoms include: skin lesions, septic shock, fever, chills and nausea. Abdominal pain, vomiting and diarrhea are less frequently reported. Death occurs in about 50 percent of the cases. A number of medical conditions make individuals more susceptible to the life threatening effects of this bacterium, including: liver disease, alcohol abuse, cancer, diabetes, chronic kidney disease, immunosuppressive drug or steroid usage, low stomach acidity and AIDS. V. vulnificus sepsis has been associated with the consumption of certain molluscan shellstock.

Hazards from *V. vulnificus* can be controlled by thorough cooking of shellfish and by preventing cross-contamination once the fish and fishery products is cooked. The risk of V. vulnificus infection may also be reduced by rapidly refrigerating oysters from the Gulf Coast during warm-weather months. Individuals in the "high risk" groups should not consume raw molluscan shellfish.

Icing is very effective to reduce the load of the organism. This organism is closely associated with oyster tissues and is not removed fully by controlled purification methods such as UV light assisted depuration. No effective means commercially exist for elimination of the health hazard in oyster intended for raw consumption and so, it is advised to avoid raw fish and fishery products completely.

Yersinia enterocolitica

Y. enterocolitica is naturally found in soil, water and domesticated and wild animals. Yersiniosis causes diarrhea, vomiting, abdominal pain and fever, often mimicking appendicitis. Outbreaks have been associated with oysters and fish.

Hazards from *Y. enterocolitica* can be prevented by: heating fish and fishery products sufficiently to kill the bacteria, holding chilled fish and fishery products below 40 F and preventing post-cooking cross-contamination.

Plesiomonas shigelloides

The genera *Plesiomonas* comprise Gram-negative, facultatively anaerobic, oxidase positive, glucose fermenting, rod shaped bacteria, generally motile. It is an emerging pathogen, mostly associated with fresh water and seawater in warm months. This organism is predominantly associated with fish and fishery products. *P. shigelloides* was implicated as the causative agent

for diarrhoea after consumption of fish and fishery products in Hong Kong and USA. It cannot grow at chilled condition, but can survive. Growth can be prevented by chilling, moderate salting/acidification.

Fungal hazards

The fungi associated with foods are generally yeasts and moulds. The greatest concern for food safety are mycotoxins eg. aflatoxin, fusarin, patulin, etc. which are produced by moulds and may be associated with chronic illness, such as cancer. Fungi needs lesser moisture for growth compared to bacteria. If the water activity (aw) is less than 0.60 there will not be any growth of fungi or other microorganisms. Water activity of biscuits is 0.30 and sugar is 0.10.

Viral Pathogens

Viruses contaminate the foods same way as bacteria. It reproduces only within susceptible living cells. A ready to eat food containing a pathogenic virus is a health hazard. Viruses don't reproduce in food; it exists in foods without growing, so they need no food, water or air to survive. Viruses don't cause spoilage but may cause illness. It can survive in human intestine, water, frozen foods etc. for months. Viruses can be found in people who were previously ill. Adequate cooking can destroy it.

Major Viral Pathogens in fish and fishery products includes:

• Hepatitis A Virus

• Norwalk Virus

Hepatitis A

This viruse survive better at low temperatures and are killed at high temperatures. As a result, most outbreaks of hepatitis occur during winter and early spring. Viruses can remain alive for long periods of time in seawater and have been shown to survive over one year in marine sediments.

Both raw and steamed clams, oysters, and mussels have been implicated in outbreaks of hepatitis A. Symptoms of hepatitis A include weakness, fever and abdominal pain. As the illness progresses, the individual usually becomes jaundiced. The severity of the illness ranges from very mild (young children often experience no symptoms) to severe, requiring hospitalization. The fatality rate is low, and deaths primarily occur among the elderly and individuals with underlying diseases.

Hepatitis A can be prevented by thoroughly cooking fish and fishery products and by preventing cross-contamination of cooked fish and fishery products. But hepatitis A appears to be more resistant to heat than other viruses. A laboratory study showed that hepatitis A viruses

in infected oysters were inactivated after heating at 140 F for 19 minutes. Therefore, mollusks steamed only until the shells open (a common cooking practice) are not exposed to heat long enough to inactivate hepatitis A viruses.

Norwalk Virus

Norwalk virus is considered a major cause of nonbacterial intestinal illness (gastroenteritis). Illness from Norwalk virus has been associated with eating clams (raw and steamed), oysters and cockles. Norwalk virus causes nausea, vomiting, diarrhea, abdominal cramps, and occasionally fever in humans.

Hazards from Norwalk virus can be prevented by thoroughly cooking fish and fishery products and by preventing cross-contamination of cooked fish and fishery products. Additionally, a recent outbreak has demonstrated that controlling overboard discharge of untreated sewage from shellfish harvesting vessels would reduce the incidence of illness attributable to Norwalk virus.

Viruses can be prevented by thorough cooking and preventing cross contamination of cooked foods.

Parasites in fish and fishery products:

Major parasites significant for human health includes:

• Anisakis simplex

• Pseudoterranova decipiens

• Diphyllobothrium latum

Anisakis simplex

Anisakis simplex, commonly called herring worm, is a parasitic nematode or roundworm. Its final hosts are dolphins, porpoises and sperm whales. The larval (wormlike) stage in fish and squid is usually 18 to 36 millimeters in length, 0.24 to 0.69 millimeters in width and pinkish to whitish in color.

Anisakiasis, the human illness caused by Anisakis simplex, is associated with eating raw fish (sushi, sashimi, lomi lomi, ceviche, sunomono, Dutch green herring, marinated fish and cold-smoked fish) or undercooked fish.

Parasites in fish are considered a hazard only in fish that the processor knows or has reason to believe will be served raw or undercooked. In other products, parasites are considered filth but not hazardous. The FDA has established three freezing processes to kill parasites. Freezing and storing at -4ºF (-20ºC) or below for 7 days (total time), or freezing at -31ºF (-35ºC) or below for 15 hours, or freezing at -31ºF (-35ºC) or below until solid and storing at -4ºF (-20ºC) or

below for 24 hours is sufficient to kill parasites. FDA's Food Code recommends these freezing conditions to retailers who provide fish intended for raw consumption. Note: these conditions may not be suitable for freezing particularly large fish (e.g. thicker than six inches).

Pseudoterranova decipiens

Pseudoterranova decipiens, commonly called "codworm" or "sealworm," is another parasitic nematode or roundworm. The usual final hosts of *Pseudoterranova* are gray seals, harbor seals, sea lions and walruses. The larval stage in fish are 5 to 58 millimeters in length, 0.3 to 1.2 millimeters in width and yellowish, brownish or reddish in color.

These nematodes are related to *Anisakis simplex* and the disease associated with infections is also termed anisakiasis. These nematodes are also transmitted to humans through raw or undercooked fish. Control of *Pseudoterranova* is the same as for *Anisakis simplex*.

Diphyllobothrium latum

Diphyllobothrium latum is a cestode, or tapeworm, that parasitizes a variety of fish-eating mammals of the northern latitudes. A similar species is found in the southern latitudes and is associated with seal hosts. Cestodes have a structure that allows them to attach to the intestinal wall of their host and have segmented bodies. Cestode larvae found in fish range from a few millimeters to several centimeters in length and are white or gray in color.

Diphyllobothrium tapeworms primarily infect freshwater fish. But salmon and related fish can also carry the parasites. Diphyllobothrium tapeworms are usually found unencysted and coiled in musculature or encysted in viscera. These tapeworms can mature and cause disease in humans. These cestodes are also transmitted to humans through raw or undercooked fish. Control of Diphyllobothrium is the same as for Anisakis simplex.

Conclusion:

Proper food handling can prevent most foodborne illness and diseases. Consumers must follow WHO's five keys to safer food -

1. Keep clean:
   - Thoroughly wash raw fruits and vegetables with tap water.
   - Keep clean hands, kitchen and chopping board all the time.
2. Separate raw from cooked:
   - Do not mix raw food and ready-to-eat food.
   - Do not mix raw meat, fish and raw vegetables.
3. Cook thoroughly:
   - Thoroughly cook all meat, poultry and fish and fishery products, especially shellfish.

- Reheat all leftovers until they are steaming hot.

4. Keep food at safe temperatures:

- Refrigerate cooked food within two hours of preparation
- Never defrost food at room temperature. Defrost frozen food in the refrigerator, cold water or in the microwave.

5. Use safe water and raw materials.

- Use safe drinking water for food preparation.
- Check use-by dates and labels while buying packed food.

Chapter 7

Molecular techniques for detection of foodborne Pathogens

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The public health concerns in recent time demands easy and accurate methods for detection of pathogens. The development of molecular techniques, new detection tools and the combination of existing approaches have increased the abilities of pathogenic bacteria monitoring and surveillance by exploring new biomarkers, increasing the sensitivity and accuracy of detection, quantification, and analyzing various genes such as functional genes and antimicrobial resistance genes (ARG). Molecular methods are gradually emerging as the most popular detection approach for pathogens, in addition to the conventional culture-based plate enumeration methods.

The field of molecular biology has a profound impact in life science investigation. Major advances in molecular biology over the last four decades have stimulated research and progress in almost all the disciplines of life science. The application of molecular technology in medicine is almost endless, some of the applications of molecular methods are:

1. Classification of organism by genotyping
2. Identification and confirmation of isolates
3. Early detection of pathogens in clinical specimen
4. Rapid detection of antibiotic resistance
5. Detection of mutations
6. Differentiation of toxigenic from non-toxigenic strains
7. Detection of microorganisms that lose viability
8. Identifying abnormalities in human and forensic medicine.

Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR), a technique used to make numerous copies of a specific segment of DNA quickly and accurately. The polymerase chain reaction enables investigators to obtain the large quantities of DNA that are required for various experiments and procedures in molecular biology, forensic analysis, evolutionary biology, and medical diagnostics.

Polymerase chain reaction (PCR) is a molecular method developed more than 30 years ago (Mullins et al. 1986) to rapidly increase copies of all or part of a DNA sequence specific to a

particular pathogen that allows further analysis of that genetic sequence. As PCR can only detect a particular DNA sequence at a time, and there are often many pathogens in a contaminated food sample, many PCR methods were developed, including multiplex PCR (mPCR), nested PCR, reverse transcription PCR, and real-time fluorescent quantitative PCR (RT-qPCR). Our paper summarizes mPCR and RT-qPCR, as they are the most widely used for the pathogen detection and identification of foodborne pathogens.

Different types of PCR:

1. Multiplex PCR
2. Nested PCR
3. Hot start PCR
4. Semi-quantitative PCR
5. Real time PCR
6. qPCR

Multiplex PCR

The principle of this technology is that multiple pairs of primers present in the reaction mixture amplify different target gene fragments in parallel. Multiplex PCR is mainly used for gene knockout, mutation analysis, and RNA detection. Thereby, this technology can improve the health and safety of food. mPCR can therefore identify many different species of pathogens that commonly contaminate food and that cause similar poisoning symptoms in humans. However, since the design of primers is the key factor in developing mPCR determination, there may be some interactions between multiple primer sets, resulting in low amplification efficiency. Therefore, primer sets should be designed with similar annealing temperature, and provide a method to distinguish amplicons after a thermal cycle .Additionally, it can lead to a false positive result as living and dead bacteria cannot be distinguished . Therefore, mPCR can often lead to unsatisfactory results.

Nested PCR

Nested PCR usually involves two sequential amplification reactions, each of which uses a different pair of primers. The product of the first amplification reaction is used as the template for the second PCR, which is primed by oligonucleotides that are placed internal to the first primer pair. In this type, increases the specificity of the amplified product for a second PCR with new primers that hybridize within the amplified fragment in the first PCR.

The increased sensitivity arises from the high total cycle number. The increased specificity arises from the annealing of the second primer set to sequences found only in the first-round

products, verifying the identity of the first-round product. In nested PCR, one of the primers in the second PCR is identical to the first.

Hot start PCR

Hot Start PCR is a technique that reduces non-specific amplification and offers the convenience of reaction set up at room temperature. The polymerases used in Hot Start PCR are unreactive at ambient temperatures. Polymerase activity can be inhibited at these temperatures through different mechanisms, including antibody interaction, chemical modification and aptamer technology. At permissive reaction temperatures reached during PCR cycling, the polymerase dissociates from its inhibitor and commences polymerization. Use of hot start DNA polymerases is most often recommended for high-throughput applications, experiments requiring a high degree of specificity, or even routine PCR where the added security offered by a hot start enzyme is desired.

Semi quantitative PCR

Fluorescent dyes like SYER Green master mix are used for the identification of samples and probes are used to measure the amount of amplified product in real- time2. cDNA is obtained by RT-PCR for a RNA sample. ApoA1/Bactin are used as markers followed by gel electrophoresis process with ethidium bromide dye staining procedure. Here the main disadvantage is the generation of non-specific hybridisation. All the reactions were performed in quadruplicates using the DNA stocks. This technique allows an approximation to the relative amount of nucleic acids present in a sample quantitative PCR.

RT-PCR

RT-PCR includes chemicals that fluoresce in the PCR reaction system. The presence of pathogenic DNA causes the mixture to fluoresce, thereby enabling pathogen presence to be monitored in real time. RT-qPCR is highly specific and sensitive. Amplified products are detected in real time without the need for post-PCR DNA analysis. For those reasons, RT-qPCR became one of the most preferred methods for detecting and identifying foodborne pathogens. The most commonly used RT-qPCR methods utilize TaqMan™ and LightCycler™ probes.

qPCR

Quantitative PCR (qPCR) is used to detect, characterize and quantify nucleic acids for numerous applications. Commonly, in RT-qPCR, RNA transcripts are quantified by reverse transcribing them into cDNA first, as described above and then qPCR is subsequently carried out. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and

elongation. However, in qPCR, fluorescent labelling enables the collection of data as PCR progresses. This technique has many benefits due to a range of methods and chemistries available.

In dye-based qPCR (typically green), fluorescent labelling allows the quantification of the amplified DNA molecules by employing the use of a dsDNA binding dye. During each cycle, the fluorescence is measured. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in "real time". The disadvantages to dye-based qPCR are that only one target can be examined at a time and that the dye will bind to any ds-DNA present in the sample.

In probe-based qPCR, many targets can be detected simultaneously in each sample but this requires optimization and design of a target specific probe(s), used in addition to primers. There are several types of probe designs available, but the most common type is a hydrolysis probe, which incorporates the use of a fluorophore and quencher. Fluorescence resonance energy transfer (FRET) prevents the emission of the fluorophore via the quencher while the probe is intact. However, during the PCR reaction, the probe is hydrolysed during primer extension and amplification of the specific sequence it is bound to. The cleavage of the probe separates the fluorophore from the quencher and results in an amplification-dependent increase in fluorescence. Thus, the fluorescence signal from a probe-based qPCR reaction is proportional to the amount of the probe target sequence present in the sample. Because probe-based qPCR is more specific than dye-based qPCR, it is often the technology used in qPCR diagnostic assay. Both the Detection and Quantification of a signal emitted by the amplified product by using the continuous measurement of a fluorescent label. It is also denoted as quantitative PCR—qPCR; usage of RT-PCR is inappropriate (Reverse Transcription PCR). Fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. Both the Detection and Quantification of a signal emitted by the amplified product by using the continuous measurement of a fluorescent label. Fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time.

APPLICATIONS OF PCR

1. Detecting pathogens using genome-specific primer pairs in food and clinical samples.
2. Detection of viral pathogens and other microorganisms persist in low levels in infected cells and are difficult to be identified by routine methods.

4. Quantitative Real-Time can be used to detect viral genomes such as HIV or HPV.

3. Diagnosis of genetic disorders such as phenylketonuria, haemophilia, sickle cell anaemia, thalassemia. Identification of genetic mutations like deletions, insertions and point mutations.

5. Screening specific genes for unknown mutations.

6. Identification and analysis of mutations in eukaryotic DNA.

7. Gene polymorphisms and Gene expression.

Advantages of PCR:

PCR is a powerful tool to amplify minute amounts of nucleic acids. Due to its unprecedented sensitivity, the method has become an essential diagnostic and research tool for infectious dermatology. Additionally, PCR can be used on all tissues or samples (fresh tissues, paraffin-embedded tissues, blood, faeces). It is also possible to analyse samples of poor conditions because only relatively short intact sequences of DNA are required. Archival materials can consequently be used for retrospective studies. In this latter case, however, amplification depends on the conservation of the target DNA. It has, for example, been demonstrated that DNA could be spoiled by long-stay in formalin and that amplification subsequently failed.

Components for PCR reaction:

The success of PCR depends on a number of factors, with its reaction components playing critical roles in amplification.

1. Template DNA
2. DNA polymerase
3. Primers
4. Deoxynucleoside triphosphate
5. MgCl2
6. Buffer

Template DNA

A PCR template for replication can be of any DNA source, such as genomic DNA (gDNA), complementary DNA (cDNA), and plasmid DNA. Nevertheless, the composition or complexity of the DNA contributes to optimal input amounts for PCR amplification. For example, 0.1–1 ng of plasmid DNA is sufficient, while 5–50 ng of gDNA may be required as a starting amount in a 50 µL PCR. Optimal template amounts can also vary based on the type of DNA polymerase used; a DNA polymerase engineered to have higher sensitivity due to

affinity to the template would require less input DNA. Optimization of DNA input is important because higher amounts increase the risk of nonspecific amplification whereas lower amounts reduce yields.

DNA polymerase

DNA polymerases are critical players in replicating the target DNA. Taq DNA polymerase is arguably the best-known enzyme used for PCR—its discovery revolutionized PCR. Taq DNA polymerase has relatively high thermostability, with a half-life of approximately 40 min at 94°C -95°C . It incorporates nucleotides at a rate of about 60 bases per second at 70°C and can amplify lengths of about 5 kb, so it is suitable for standard PCR without special requirements. Nowadays, new generations of DNA polymerases have been engineered for greatly improved PCR performance.

For more specialized applications such as PCR cloning, long amplification, and GC-rich PCR, DNA polymerases with higher performance are preferred. These enzymes are capable of generating lower-error PCR products from long templates in a shorter time with better yields and higher resistance to inhibitors (learn more about DNA polymerase characteristics).

Primers

PCR primers are synthetic DNA oligonucleotides of approximately 15–30 bases. PCR primers are designed to bind (via sequence complementarity) to sequences that flank the region of interest in the template DNA. During PCR, DNA polymerase extends the primers from their 3′ ends. As such, the primers' binding sites must be unique to the vicinity of the target with minimal homology to other sequences of the input DNA to ensure specific amplification of the intended target.

In addition to sequence homology, primers must be designed carefully in other ways for specificity of PCR amplification. First, primer sequences should possess melting temperatures (Tm) in the range of 55–70°C, with the Tm of the two primers within 5°C of each other. Equally important, the primers should be designed without complementarity between the primers (especially at their 3' ends) that promotes their annealing (i.e., primer-dimers), self-complementarity that can cause self-priming (i.e., secondary structures), or direct repeats that can create imperfect alignment with the target area of the template.

Furthermore, the GC content of the primer should ideally be 40–60%, with uniform distribution of C and G bases to avoid mistakes. Similarly, no more than three G or C bases should be present at the 3′-ends of the primers, to minimize nonspecific priming. On the other hand, one C or G nucleotide at the 3′ end of a primer can promote beneficial primer anchoring and

extension. For convenience and simplicity, a number of online tools are available to bioinformatically design and select optimal primer sequences with defined parameters.

Higher primer concentrations often contribute to mispriming and nonspecific amplification. On the other hand, low primer concentrations can result in low or no amplification of the desired target.

Deoxynucleoside triphosphates (dNTPs):

dNTPs consist of four basic nucleotides—dATP, dCTP, dGTP, and dTTP—as building blocks of new DNA strands. These four nucleotides are typically added to the PCR reaction in equimolar amounts for optimal base incorporation. However, in certain situations such as random mutagenesis by PCR, unbalanced dNTP concentrations are intentionally supplied to promote a higher degree of misincorporation by a non-proofreading DNA polymerase.

In common PCR applications, the recommended final concentration of each dNTP is generally 0.2 mM. Higher concentrations may help in some cases, especially in the presence of high levels of Mg2+, since Mg2+ binds to dNTPs and reduces their availability for incorporation. However, dNTPs exceeding optimal concentrations can inhibit PCR. For efficient incorporation by DNA polymerase, free dNTPs should be present in the reaction at a concentration of no less than 0.010–0.015 mM (estimated Km). When using non-proof reading DNA polymerases, fidelity can be improved by lowering dNTP concentrations (0.01–0.05 mM), as well as proportionally reducing Mg2+.

Magnesium ion (Mg2+):

Magnesium ion (Mg2+) functions as a cofactor for activity of DNA polymerases by enabling incorporation of dNTPs during polymerization. The magnesium ions at the enzyme's active site catalyse phosphodiester bond formation between the 3′-OH of a primer and the phosphate group of a dNTP. In addition, Mg2+ facilitates formation of the complex between the primers and DNA templates by stabilizing negative charges on their phosphate backbones.

Mg2+ ions are commonly delivered as a MgCl2 solution to the PCR mixture. However, some polymerases such as Pfu DNA polymerase prefer MgSO4, since sulfate helps ensure more robust and reproducible performance under certain circumstances. The magnesium concentration often needs optimization to maximize PCR yield while maintaining specificity due to its binding to dNTPs, primers, DNA templates, and EDTA (if present).

Buffer:

PCR is carried out in a buffer that provides a suitable chemical environment for activity of DNA polymerase. The buffer pH is usually between 8.0 and 9.5 and is often stabilized by Tris-

HCl. For Taq DNA polymerase, a common component in the buffer is potassium ion (K+) from KCl, which promotes primer annealing. At times, ammonium sulphate (NH4)2SO4 may replace KCl in the buffer. The ammonium ion (NH4+) has a destabilizing effect, especially on weak hydrogen bonds between mismatched primer-template base-pairing, thereby enhancing specificity (Figure 8). Note that DNA polymerases often come with PCR buffers that have been optimized for robust enzyme activity; therefore, it is recommended to use the provided buffer to achieve optimal PCR results.

Since Mg2+ has a stabilizing effect similar to K+, the recommended MgCl2 concentrations are generally lower when using a KCl buffer (1.5 ± 0.25 mM) but higher with an (NH4)2SO4 buffer (2.0 ± 0.5 mM). Due to antagonistic effects of NH4+ and Mg2+, buffers with (NH4)2SO4 offer higher primer specificity over a broad range of Mg2+ concentrations. It is important to follow buffer recommendations by the enzyme's supplier, since the optimal PCR buffer is dependent upon the DNA polymerase used.

VISUALIZATION OF PCR TEST RESULTS:

Amplicons visualization in post PCR process, the template DNA is usually done by following-

Agarose Gel Electrophoresis-

After PCR is performed, the result can be analysed by agarose gel electrophoresis. This technique will allow a "visualization "of the PCR amplification reaction regarding the number of amplified regions (one or more PCR products) and the size of the PCR products (the size is the expected one based on the predicted amplicon flanked by the primers). Agarose gel electrophoresis separates DNA molecules according to size.

First, a board of agarose gel is assembled with wells (depressions) on top. The gel is submerged in electrophoresis buffer inside the electrophoresis tank. The PCR products (DNA) are then deposited on the wells and an electrical current is passed through the gel, conducted by the salts in the electrophoresis buffer (e.g. TAE - Tris-acetate-EDTA electrophoresis buffer).

Because DNA has a negative charge the current drives the DNA across the gel into the positive electrode. The agarose gel is a porous matrix where DNA molecules move according to size; smaller molecules take less time to move towards the end of the gel because shorter DNA fragments are not retained by the pores of the gel. Therefore, when we have a mixture of different DNA molecules, shorter molecules will migrate faster and will be deposited as bands at the bottom of the gel. Longer DNA molecules will be deposited as bands at the top of the gel because they have been retained by the pores in the gel matrix.

Before applying the DNA into the wells of the gel, the DNA is mixed with loading buffer This buffer is composed of glycerol (to enable the deposition of the DNA inside the wells) and bromophenol blue dye (to visualize the DNA solution when loading the samples and monitoring the progression of the electrophoresis run). The loading buffer does not interfere with the electrophoresis.

After the current is stopped, the DNA bands can be visualized using a fluorescent stain that intercalates the DNA molecules and a UV light equipment. There are several types of stains that intercalate the DNA molecules, emitting fluorescence when the gel is irradiated under UV light (254nm -365 nm) such as ethidium bromide (EtBr), SybrGreen or GelRed. These agents are added to the agarose when assembling the gel

Contaminants and inhibition

Sample quality is critical and therefore it must be verified before the samples are used in qPCR assays. It has been clearly demonstrated that analysis of degraded RNA samples can result in poor quality. In addition, it has been shown that the presence of inhibitors differentially affects qPCR assays. Therefore it is important to determine the integrity and purity of nucleic acid samples. contamination refers to the presence of an unwanted component in a sample. Classic examples of contamination in biological research experiments include the presence of mycoplasma in cell culture or unwanted proteases during serological analysis. Within the context of qPCR assays, the significant forms of contamination refer to nucleic acid template present in the sample which may be detected along with the specific target, thus generating a false positive, or material that may inhibit downstream reactions resulting in a reduced or failed reaction (false negative). Amplicon (target sequence) contamination PCR assays are especially vulnerable to contamination with the specific target sequence of interest for two reasons: PCR is a process that generates billions of copies of the amplicon, the specific target of interest. However, amplified product from one PCR is potentially a source of contamination for future PCRs. In a molecular biology laboratory, it is therefore essential to separate the PCR set up area (the designated space in which the reaction is prepared) from the post-PCR analysis area (the site the product will be subsequently analysed and possibly manipulated). This should be achieved by using separate rooms with dedicated equipment and laboratory coats such that nothing from the post-PCR analysis space is brought into contact with the clean (pre-PCR) space. Many laboratories also introduce a one way policy such that if an individual has entered a post-PCR room they are forbidden from subsequently entering a prePCR room on the same day, and qPCR assays are particularly vulnerable to amplicon contamination given the scope

of detecting a single template molecule; hence minuscule amounts of contamination are sufficient to cause a problem

In addition to PCR generation of the specific amplicon, care is needed in the laboratory setup to ensure that template material is not transferred from one sample to another (i.e. cross contamination). In the case of analysis of human samples, there must be precautions to ensure that material is not introduced from the analyst. It is important to identify and avoid contamination of all samples, and the use of controls for this purpose is highly recommended

CONCLUSION

The invention of PCR and real-time PCR has led to many major scientific advances. Though both methods are still regularly used in laboratories, real-time PCR is gaining popularity and quickly becoming the most cost- and time-effective method for analysing DNA products.

The use of real-time PCR expands to many areas of the clinical laboratory including genetics, virology, and microbiology. With more real-time PCR platforms and practices being created, the growth and potential of this technology is just beginning. However, the concept and process will stay the same and it is important for laboratory professionals to understand and learn about this technology.

Chapter 8

Quality Issues in Fish and Fishery Products

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Introduction

Fish is a nutrient rich food commodity with high perishable nature. It is highly susceptible to post harvest losses both physical (material) and quality. According to ISO, Quality is defined as the totality of features and characteristics of a product or service that bears its ability to satisfy stated or implied needs. Quality loss of fish refers to the undergone changes due to spoilage or physical damage and finally resulted quality deterioration. Quality and safety are important parameters for perishable foods like fish and fish products. Directly after death of the fish, a series of biochemical reactions starts, which is of paramount importance for the quality and shelf life of products.

Spoilage of fish

Spoilage is defined as the sensory changes resulting in a fish product being unacceptable for human consumption. Fresh product is defined as the one whose original characters remain unchanged. Spoilage therefore is the indicative of post-harvest change. This change may be graded as the change from absolute freshness to limits of acceptability to unacceptability. Spoilage of fish begins immediately after the death of the fish. Fresh fish spoilage can be very rapid after it is caught. The spoilage process will start within 12 h of their catch in the high ambient temperatures of the tropics. It depends upon several different factors such as the type of fish species, the physiological condition of the fish, as well as environmental influences (for example water temperature, salinity). In addition, catching and harvesting methods, killing procedures and the performance of slaughtering have a great effect on the biochemical reactions. The spoilage of fish is a complicated process brought about by actions of enzymes, bacteria and chemical constituents. The process involves three stages namely enzymatic autolysis, oxidation and microbial spoilage.

1. Enzymatic spoilage - A number of proteolytic enzymes are found in muscle and viscera of the fish after catch. These enzymes contribute to post mortem degradation in fish muscle and fish products during storage and processing. There is a product associated alteration that can be contributed by proteolytic enzymes. Lactic acid produced by the anaerobic glycolytic pathway in post mortem fish decrease the pH from 7 – 7.2 to 6.2 – 6.5 resulting post mortem stiffening called rigor mortis. Autolytic enzymes activate nucleotide degradation process and result bitter tasting hypoxanthine which is an indicator of loss of freshness. Proteolytic enzymes cause extensive autolysis which results in meat softening and rupture of the belly wall.
2. Microbial spoilage - Directly after the catch, the muscle tissue of healthy marine fish is free from bacteria, but not the gills, skin and intestines. The bacteria penetrate into the fillet mainly through the gills and body cavity during storage and processing, accompanied by changes in the composition of the bacterial flora. Gram-negative psychrotrophic rods (*Shewanella* spp., *Pseudomonas* spp., *Vibrio* spp. and *Aeromonas* spp.) are important spoilage bacteria. Microbial growth and metabolism is a major cause of fish spoilage which produce amines, biogenic amines such as putrescine, histamine and cadaverine, organic acids, sulphides, alcohols, aldehydes and ketones with unpleasant and unacceptable off-flavors.
3. Chemical spoilage - Lipid oxidation is a major cause of deterioration and spoilage for the pelagic fish species such as mackerel and herring with high oil/fat content stored fat in their flesh. Lipid oxidation involves a three stage free radical mechanism: initiation, propagation and termination. Oxidation typically involves the reaction of oxygen with the double bonds of fatty acids. Therefore, fish lipids which consist of polyunsaturated fatty acids are highly susceptible to oxidation. In fish, lipid oxidation can occur enzymatically or non enzymatically. The enzymatic hydrolysis of fats by lipases is termed lipolysis (fat deterioration).

Quality issues in chilled fish and shellfish

- **Belly bursting –** Belly bursting of fish is due to enzymic spoilage in well fed fish. It can occur in only a few hours after catch in sardines, herring etc, is caused simply by a weakening of the belly wall due to self-digestion. The rate of self-digestion is much dependent on temperature. Chilling of the fish to just above the freezing point does not stop, but retards self-digestion. In the dissolved gut components, bacteria proliferate

and produce gases such as CO_2 & H_2. This gas production leads to belly bursting after short storage period.

- **Blackening or melanosis –** Black spot or mealnosis is objectionable black discolouration appearing in crustaceans especially shrimps, lobsters etc during storage. Dark pigments mainly appear in the cephalothorax and joints region, which is due to the enzymatic oxidation of phenolic compounds by poly phenoloxidase (PPO) enzyme. Even though it is harmless, it reduces marketability of shrimp. Sulphite containing compounds namely sodium metabisulphate at 0.2 to 0.5 % (w/v) for one minute dip treatment is used in industries to prevent blackening of shrimp.
- **Discoloration -** Darkening of dark-fleshed fish such as sardine and mackerel during iced storage is mainly caused by the formation of metmyoglobin by the auto oxidation of myoglobin. The discoloration increase with the increase of storage period.
- **Pink discoloration in squid and cuttle fish –** Pink discoloration in squid during handling and chilled storage reduce the quality and acceptability. Pigment released from the disrupted chromatophores localized in the skin most likely stains the mantle during the handling or storage. Large squids are more prone to develop colour in comparison to small. Pink colouration is commonly observed in squid stored in insufficient ice as well as stacking condition for extended period. Bleaching agents are commonly used to prevent pink discolouration.

Quality issues in frozen fish

- **Freezer burn or dehydration –** Freezer burn or white patches develop on the surface of frozen stored fish due to vapour pressure gradient within the product and environment during temperature fluctuation. High temperature of material while loading, frequent opening of the door, excess defrosting and high air velocity accelerate dehydration. Proper packaging without air pockets, sufficient glazing, constant storage temperature and relative humidity are important for controlling this defect. The critical limit of dehydration is $50 g/m^2$/day.
- **Yellow discoloration -** Yellowish color in the flesh, occurs in some frozen fish as a result of chromatograph disruption with consequent release and migration to the subcutaneous layer. Yellow discolouration reported in frozen pomfrets is due t

oxidation. Application of glaze water containing antioxidants can prevent yellowing of frozen pomfrets.

- **Green discoloration in frozen tuna –** It is a common defect in frozen tuna and sword fish. Hemoproteins react with hydrogen sulphide produced from deterioration of fish under aerobic condition to form sulfhaemoglobin and sulfmyoglobin. These are green pigments accompanied with sour smell and off flavor. It can be prevented by proper bleeding of fish and maintenance of iced condition until frozen.
- **Protein denaturation –** Fluctuation in the frozen storage temperature leads to protein denaturation. It results loss of functional property and textural damage.
- **Weight loss –** frozen fish loss weight upon thawing as thaw drip. Along with thaw drip many water soluble nutrients and flavor are lost. Increased amount of thaw drip affect the appearance of the product and result weight loss. Weight loss increase with pre freezing ice storage period.

Quality issues in dried fish

- **Pink formation -** The formation of pink or red discolouration on surface of the cured or salted product adversely affects the appearance. Halophilic bacteria species of the genus *Halobacterium* and *Halococcus* attack dried fish and a pink or red discolouration is formed. Spoilage appears on the surface as slimy pink patches. They are aerobic and proteolytic in nature, grows best at 36ºC by decomposing protein and giving out an ammoniacal odour. However, these bacteria are not harmful in nature. Usage of good quality salt is recommended to avoid this condition.
- **Dun formation -** Dun is a brown or chocolate coloured pepper like spot that grows in dried fish at 10 to 15% salt content. This is mainly caused by growth of halophilic mould called *Sporendonema epizoum. Wallemia spora* and *Wallamia sabi* appears chocolate in colour. The most common species, *Aspergillus species* and *A. glaucus species* can cause an objectionable flavour and textural changes in fish. The moulds are harmless, do not damage the flesh and growth is very slow. It can be prevented by good hygienic method in and around the processing plant. The presence of mould on the surface of the fish makes the product unacceptable to the consumer. To avoid the mould growth it is necessary that the fish be dried, packed and stored properly to avoid uptake of moisture.

- **Salt Burn:** A mixture of large and small grain sizes is recommended for dry salting of fish. If fine grain is used directly on the fish, salt burn may occur due to the rapid removal of water from the surface with no penetration of salt to the interior of the fish.
- **Chemical impurities of commercial salt:** main chemical impurities in salt are calcium chlorides and sulphates, magnesium chlorides and sulphates, sodium sulphate and carbonate and traces of copper and iron. Calcium and magnesium chlorides slow down the penetration of salt into the fish, thus increasing the spoilage rate. Magnesium chloride is hygroscopic in nature and tends to absorb water thus making the more difficult to dry. Excessive quantities of calcium and magnesium compounds impart a bitter taste to the fish and make it brittle when dry. Traces of copper gives a brown appearance to the fish making it look spoiled.
- **Case hardening –** When the rate drying is very rapid due to high temperature and low relative humidity, the surface of the fish can become very hard or 'case hardened'. This prevents the movement of moisture from the deeper layers to the surface. The surface of fish will be dried, while the centre remains wet and hence spoils quickly.
- **Rancidity:** This is caused by the oxidation of fat, mostly in oil rich fishes like mackerel. Oxidation of fat imparts characteristics odour and colour of the fish change to brown. This is known as rust. Certain impurities in salt and traces of copper accelerate this.
- **Insect infestation:** Dried fish losses in many developing countries are caused by insect infestation. The flies which attack the fish during the initial drying stage are mainly blowflies belonging to the family Calliphoridae and Sarcophagidae. These flies are attracted by the smell of decaying matter and odours emitted from the deteriorating fishes. During the glut season when the fish is in plenty and some are left to rot, these flies come and lay their eggs. These eggs develop into maggots, which bury within the gill region and sand for protection from extreme heat and develop mainly when conditions are favourable. The most commonly found pests during storage are beetles belonging to the family Dermestidae. Beetles attack when the moisture content is low and especially when the storage is for a long time. The commonly found beetles are *Dermestes ater, D frischii, D maculates, D carnivorous* and *Necrobia rufipes*. The larva does most of the damage by consuming dried flesh until the bones only remain. Mites are also an important pest, which are found infesting dried and smoked products. *Lardoglyphus konoi* is the commonly found mite in fish products. Infestation can be reduced by proper hygiene and sanitation, disposal of wastes and decaying matter, use

of physical barriers like screens, covers for curing tanks etc, and use of heat to physically drive away the insects and kill them at 45 º C.

- **Fragmentation:** Denaturation and excess drying of fish results in breaking down of the fish during handling. Fish can become brittle and liable to physical damage when handled roughly. Insect infestation is also a reason behind fragmentation in dried samples. It is necessary that fresh fish be used as raw material to ensure a good finished product.

Quality issues in canned fish products

- **Discoloration –** It is normally observed in canned shellfishes. It can be result of physical, chemical or microbiological activity.
- **Blackening** of the contents or inside of the can is most often encountered in packing crab, clams, shrimp and lobster, but may also be found in other canned products. It occurs most readily where the product has an alkaline reaction. Sulfur compounds in the flesh of these species break down in processing and unite with the ion base of the tin plate to form iron sulfide. Parchment paper linings, the use of organic acids and lacquer coatings can prevent this problem.
- **Copper sulfide discoloration** is associated with hemocyanin, a biochemical component in crustacean blood. This is often the result of protein sulphur compounds breaking up under high temperature during blanching or cooking, and their combining with iron forming black iron sulphide. The use of enamel lined cans for these products eliminates this problem.
- **Can corrosion -** Internal can corrosion can lead to the accumulation of hydrogen which relieves vacuum and swells the can making it unmarketable. Externally, corrosion often causes pinholes that allow micro-organisms to penetrate the can and spoil its contents.
- **Stack burning –** It is a type of discoloration due to over processing. A considerable amount of heat is retained over a long period when canned products are stacked or cased before they are sufficiently cooled. Cooking goes on over a much longer period than is intended, which affects both color and flavor unfavorably.
- **Microbial spoilage -** Bacterial spoilage in canned fish and shellfish is caused almost entirely by organisms of high heat resistance, and may be divided into two general types, gaseous and non-gaseous. Swelled or "bulging" can ends are a common indication of gaseous decomposition. Organisms found quite commonly are

Clostridium welchii and *Clostridium sporogens.* Gas formation is accompanied by an extremely foul and offensive odor. Gas-forming heat-resistant organism is *Clostridium botulinum.* Spoilage may not always be accompanied by the excessively disagreeable odor. There is no external indication of non-gaseous spoilage. The ends of the containers are flat and the contents may be normal in appearance. An "off" odor mayor may not be noticeable, but the product is sour in taste. This type of spoilage is known as "flat souring." It is caused by aerobic spore formers *Bacillus cereus, B. mesentericus* and *B. vulgatus.*

- **Bulged (swollen) cans –** They are **Flipper**, **Springer**, **Soft swell** and **Hard swell.** Flipper is the initial stage of swell and in flipper, can which may be normal in appearance, but if one end is struck on a box or table, the other end becomes convex, though the convexity may be pressed down again. Reasons are under exhausting, 1st stage of H2 production, 1st stage of microbial activity or pre-processing spoilage of food. In springer, a can having convex or bulging ends, which may be pressed flat, again with the fingers, but will spring out again after pressure is released. Reasons are same as that of flipper. In soft swell, permanently convex can ends. But when pressed by finger it gets depressed but when the pressure is removed it regains original bulge. In hard swell permanently convex ends and do not get depressed due to pressure by fingers. Soft and hard swell are due to high pressure gases, more hydrogen production or advance bacterial reaction.
- **Flat sour -** A can whose contents may be spoil d by microbiological action without the formation of gas and no external indication of spoilage. The product has a sour taste and may or may not have sour odour.
- **Leaker spoilage -** The micro-organisms involved in leaker spoilage can be any type found on can-handling equipment, in cooling water or on the skin of cans handlers. These include bacterial cocci, short and long rods, yeasts and moulds, aerobic sporeformers or, more likely, a mixture of many of these organisms. Postprocess contamination can also result in outbreaks of botulism or *Staphylococcus* enterotoxin poisoning. Leaker spoilage is often associated with the integrity of the can seams, the presence of bacterial contaminants in the cooling water or on wet can runways and abusive can-handling procedures after heat processing. Cooling water can be the primary source of organisms responsible for leaker spoilage.

- **Honeycombing -** Honeycombing is found in canned tuna meat that is processed from stale raw material. The meat in such cases presents the appearance of honeycomb. During steaming the volume of the meat will contract due to removal of water because of the coagulation of muscle protein that begins at the surface. Production of gas in the flesh expands and makes little pockets in the flesh. On cooling, the pockets remain and the flesh seems to be filled with small holes or air paces. It also occurs in canned salmon and sardines.
- **Mush -** It is flabby condition met with some species of pilchards caught at the end of its spawning. This is caused by the invansion of prasitic protozoan *chloromyxum* whish decomposes the fish meat during storage such that it becomes entirely soft during canning.
- **Struvite formation -** Canned marine products such as brine packed shrimp,crab, tuna,salmon etc.are frequently seen to contain some glass like crystals,particulary when the temperature of storage is low. It occurs due to the formation of a chemical compound ,magnesium ammonium phosphate hexahydrate,MgNH4PO46H2O, called struvite. It can be prevented by adding chelating agent like hexametaphosphate.
- **Curd -** 'Curd' is precipitated protein often found in canned mackerel and salmon. This is more common with salmon, which is generally canned without pre-cooking. The meat coagulated by heat adheres to the inner side of the can ends and presents a poor appearance on opening the can. The lacquer may get peeled off while removing the curd from the can ends. Use of raw fish, which is not very fresh, and, inadequate brining and pre-cooking are some of the reasons responsible for formation of curd. It can be prevented if the raw fish is soaked in 10-15% brine for 20-30 minutes followed by thorough washing before filling.

Quality issues in minced fish products

- **Dehydration** - Deep Dehydration Greater than 10% of the surface area of the sample unit exhibits excessive loss of moisture clearly shown as white or yellow abnormality on the surface which masks the colour of the flesh and penetrates below the surface, and cannot be easily removed by scraping with a knife or other sharp instrument without unduly affecting the appearance of the block.
- **Foreign Matter -** The presence in the sample unit of any matter which has not been derived from fish (excluding packing material), does not pose a threat to human health,

and is readily recognized without magnification or is present at a level determined by any method including magnification that indicates non-compliance with good manufacturing and sanitation practices.

- **Parasites -** The presence of two or more parasites per kg of the sample unit detected with a capsular diameter greater than 3 mm or a parasite not encapsulated and greater than 10 mm in length.
- **Bones (in packs designated boneless) -** More than one bone per kg of product greater or equal to 10 mm in length, or greater or equal to 1 mm in diameter; a bone less than or equal to 5 mm in length, is not considered a defect if its diameter is not more than 2 mm. The foot of a bone (where it has been attached to the vertebra) shall be disregarded if its width is less than or equal to 2 mm, or if it can easily be stripped off with a fingernail.
- **Odour and Flavour** - A sample unit affected by persistent and distinct objectionable odours or flavours indicative of decomposition or rancidity or of feed.
- **Flesh abnormalities** - A sample unit affected by excessive gelatinous condition of the flesh together with greater than 86% moisture found in any individual fillet or a sample unit with pasty texture resulting from parasitic infestation affecting more than 5% of the sample unit by weight.

Quality issues in battered and breaded products

- **Voids** – It is a common quality problem. Voids are bare areas that do not accept batter. It is due to excessive line speed, shape of fish portion, absence of pre dusting material, a no adhesive surface, ice glaze and air pockets. Once it is formed it is difficult to remove
- **Blow off** – This is seen when some or all of the batter is blown off or removed during frying. This is accelerated if it contains voids. It gives a dark unacceptable appearance.
- **Pillowing** – It is an elevated dome of batter on the product with a large air pocket beneath it. It is caused by the formation of steam pocket due to water vapourization which is trapped under the batter during the frying process.
- **Tailings** – Batter extends beyond the product like a tail or stringer. It is caused due to the excessive thick batter which results in inadequate blow off during the production.

Chapter 9

Sanitation and hygiene in seafood processing

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Seafood focused facilities require more care than others as they demands special storage needs, which makes sanitation in fish processing plant facilities even more critical. Taking the proper steps to ensure a sanitized facility will create a safe environment to produce food products for the public. The better you maintain your facility, the better the products you produce will be, and there will be less risk for contamination.

Due to the popularity of seafood, the need for fish processing plants has increased significantly. Fish processing plants deal with water more than any other food processing plants, increasing the concern about bacteria and contamination within the facility. Ensuring sanitation in fish processing plant facilities is critical.

Follow these steps to help ensure maximum sanitation levels and prevent a serious crisis:

1. Water Safety

Water is an essential component of fish processing plants, and water safety covers many areas. Water used for food and food-contact surfaces, producing ice, and separating potable and non-potable water sources is vital for facility sanitation. Any water should come from government-approved sources to ensure it is safe for food, food-safe sources, and human consumption. Monitoring is another essential part of water safety to ensure sanitation levels of potable water remain steady and any cross-connections don't lead to contamination.

2. Condition and Sanitation of Food Contact Surfaces

Anything that comes into contact with food products during production counts as a food contact surface. Keeping these surfaces clean is vital. Maintaining them minimizes the potential of them needing repairs or replacing. To ensure you aren't damaging the surface, you want to watch what products you use and the concentration level of the chemicals. The easiest way to

avoid issues is to choose a material like stainless steel, which is resistant to bacteria and corrosion.

Inspect surfaces regularly, including gloves and outer clothing, to ensure it is in good condition, clean, and protecting the employees. With cleaners and sanitizers, test the chemical levels of the products to ensure they aren't too strong.

Fish muscle usually contains no bacteria, so if it's cut with a knife or is in contact with an infected surface after cutting, the food poisoning bacteria may be the first arrivals. Food Poisoning is preventable if we keep food clean, cool, and free from contamination at all times during handling.

3. Condition and Maintenance of Employee Hygiene Areas

Employee hygiene is a critical part of any food processing plant. Hand washing, hand sanitizing, and toilet facilities are all required to help maintain the sanitation in fish processing facilities. You want to routinely monitor the condition of these facilities, ensuring they are clean and functioning properly. You want to repair toilets that are not working, keep all necessary supplies replenished, and ensure hand sanitizer meets the necessary concentration levels. Have all of these areas assessed by someone familiar with the requirements to ensure that nothing gets overlooked.

4. Store, Label, and Use Toxic Materials Properly

Even though a fish processing plant deals with food, toxic chemicals are kept on the premises. Cleaners, sanitizers, insecticides, and lubricants for the equipment are just some of the chemicals that can be found. These chemicals are necessary for various reasons, but it is vital to take the proper precautions to ensure the fish and production area remain safe and free from harmful chemicals.

To prevent any dangerous situations, you need to take the time to organize, sort, store, and label all chemicals properly. Containers should be durable enough to handle the chemicals, especially if they are corrosive, and labels should be clear, providing the name, manufacturer information, and instructions for use. These chemicals should also remain away from food products and production equipment to prevent potential contamination or other damage.

5. Pest Control

Pests refer to more than just rodents; it includes birds, insects, and other animals. Any pests inside a facility are incredibly problematic. They can create contamination, spreading bacteria like Salmonella, Staphylococcus, and Listeria, to name a few. Contamination can lead to a significant loss of revenue and cause damage to the company's reputation.

You need to ensure there are no entry points for pests and that deterrents are in place outside of the facility. Keeping the facility clean and free from food debris is also critical so that there is nothing to draw pests to the facility. Even with preventive measures in place, it is important to perform regular inspections to ensure the facility remains pest-free and take proper action if there are any concerns, to prevent a serious infestation.

6. Install a Sanitary Drainage system

Floor drainage is a critical part of the sanitation in fish processing plant facilities. These systems help with water safety by keeping water from pooling on the ground, eliminating the risk of bacteria growing. By keeping floors clean, floor drains also help with pest control.

Food Safe Drain's industrial strength 10,000 Series Slot Drain is the perfect addition to any facility. The drain features a seamless design with NSF-certified T304 or T316 stainless steel. A grate-free design means that inspectors can look at the drain without anything blocking their view–it also makes maintenance much easier, especially with the inclusion of a clean-in-place system.

Requirements for an effective sanitation programme

• Implementation of effective sanitation programme becomes necessary in any food processing industry. For a workable sanitation programme the following requirements have to be met. Management must be aware of the need for good sanitation.

•The processing unit should be suitably located and constructed.

•The processing unit must have required quantity of good quality water supply.

•The processing unit must have adequate washing facility for whole fish.

•The processing unit must have smooth working surfaces.

•The processing unit must have a sound clean-up policy.

•The processing unit must have adequate sanitary facilities.

•The processing unit must ensure good personal hygienehabits of employees.

• The processing unit must have effective rodent and insect control programme.

Need for hygienic practices:

• Practicing of proper hygienic practices by food handlers is of critical importance as they serve as fundamental sources of many microorganisms responsible for foodborne illnesses.

• Pathogens such as Staphylococcus aureus is naturally associated with humans, and will contaminate the food unless proper care is taken. Production of toxin by this organism in any food is responsible for staphylococcal food poisoning.

• The food handlers could also serve as carriers for many of the pathogenic microorganisms responsible for typhoid fever, dysenteries, salmonellosis, hepatitis etc.

• So, there is a need for acceptable hygienic practices by food handlers to prevent microbial contamination to food.

• Compliance to proper hygienic practices by the food handlers can be achieved only by educating them on the importance and need for personal hygiene practices.

Clean- up procedure

• The absolute cleanliness of personnel, equipment and premises is very important at all stages of food production.

• The main objective of cleaning is to minimize the risk of cross contamination between foods and also from waste food material left on equipment and food contact surfaces.

• The clean-up procedure in food processing industry assures; Protection of food from contamination by pathogenic microorganisms that can cause disease in consumers.

• Protection of food from spoilage organisms that can cause deterioration in the quality of food.

• Maintenance of good standards of cleanliness which helps minimizing the risk of rodent and insect infestation.

• A good clean-up procedure generally consists of initial cleaning procedure followed sanitation phase.

Cleaning phase

Cleaning can be defined as the removal of all dirt, slime, blood, oil, grease and any food soil from all food contact surfaces.This operation is accomplished with the aid of a detergent which when added to water helps to remove all kinds of dirt effectively. The basic steps involved in

cleaning phase are initial rinsing with cold water followed by scrubbing with warm water and detergent, and final rinsing with cold water.

Sanitizing phase

Sanitizing or disinfecting phase is the parts of the cleaning operation wherein surfaces and equipment are rendered microbiologically clean by using sanitizers. Sanitizing is done after cleaning phase and it involves cleaning using sanitizers followed by final rinse with cold water.

• The cleaning operation involves separation of dirt/soil from the object to be cleaned, the dirt away and its suitable disposal using detergent, leaving the surface being cleaned in the desired condition and disinfection when desirable.
• Selection of a suitable cleaning solution or detergent is necessary to achieve effective cleaning as it depends on various factors such as neutralization, pH, alkalinity, buffering capacity, surface tension, wetting ability, mechanical action, emulsification, deflocculation, etc.
• Several types of mechanical aids are available for use in food industry for cleaning and sanitation purpose.

Guidelines for the use of sanitizers

The following guidelines should be followed in attaining effective sanitation in a food industry. Sanitizer should never be used as a substitute for thorough cleaning. Sanitizers should be used as an additional safeguard to thorough cleaning.Should not be used where sterilization need to be achieved. Because, sterilization aims at destroying or removing all microbial life, whereas sanitization does not kill all microorganisms, but help to reduce microbial load. The detergents used for cleaning purpose should be inexpensive, approximately neutral (pH 6-8), biologically degradable, easy to wash away, non-toxic and approved by the appropriate regulatory body for food use. The sanitizer used in the food industry should possess good bactericidal activity and active against a wide variety of organisms, be non-toxic or low in toxicity, not affect the colour, odour and flavour of the food, be fairly cheap, be easy to dispense and wash away, should not adversely affect the operators and food handlers and should not cause damage to surfaces being sanitized (Ex.equipments). Common sanitizing agents
•The most commonly used sanitizing agents in food industry arechlorine, iodine and phenolic compounds. Chlorine is morecommonly used than the other two in food processing plants.
• It is the least expensive and readily available in several forms. Iodine is more expensive and is not easily available, but is more effective than chlorine even in low concentrations.

• Phenolic compounds are not used in fish processing plant as their use even in very small quantities leaves a long-lasting odour and bad taste.

Degree of cleanliness

• Equipment, object or any food contact surface is considered clean onlywhen it is clean physically, chemically and microbiologically.

• The object or the surface is considered physically clean when it appears clean by sight and feel. This is easy to achieve as this can be easily tested by close observation.

• Chemical cleanliness of an object or surface refers when the object or surface is free from traces of undesirable chemicals (too small to be seen or felt, but sufficient to influence the quality of the product).

• Even traces of some chemicals can affect the taste, odour, colour or keeping quality of the food product.

The object or surface is considered microbiologically clean when it is free from undesirable microorganisms. This kind of cleanliness can be tested by determining the kind and number of microorganisms left on the object cleaned, decrease or increase of bacterial load of the product at the end of the processing line, and keeping quality of the product

Chapter 10

Analysis of histamine in fish and fish products

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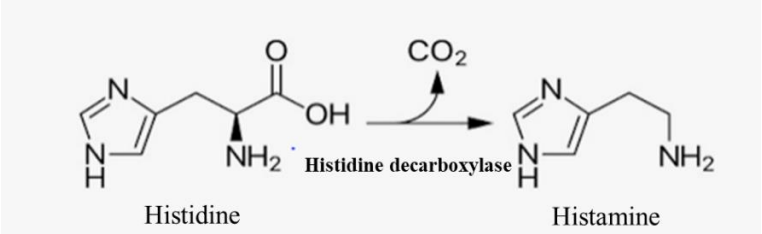
Introduction

Histamine is formed as a result of time-temperature abuse of certain fish species and can cause illness to the consumer. The illness is closely linked to the growth of bacteria capable of producing histidine decarboxylase enzyme to develop histamine in those fish. Food safety and standards regulation (FSSR) of India had set the maximum limit for histamine at 200 mg/kg for fishery products with an exception in a fish pickle, dried/salted, and fermented fish which is set at 400 mg/kg. Histamine-related illness in seafood is also known as scombrotoxin poisoning, which is primarily associated with the consumption of fish such as tuna, mackerel, bonito, etc. Other fish, such as mahi mahi, bluefish, marlin, anchovy, etc. can also cause scombroid fish poisoning. Originally, the illness was termed "scombroid poisoning" because of its association with fish in the families Scombridae and Scomberesocidae. However, other species of fish are now known to cause the illness and hence the terms "histamine poisoning" and "histamine fish poisoning" have also been applied to the illness. However, biogenic amines other than histamine are also known to have associated with the illness.

Process of histamine formation

Histidine decarboxylase enzyme is produced by certain bacteria during their growth. This enzyme reacts with histidine present in larger quantities in some fish than in others. The result is the formation of scombrotoxin (histamine).



Histamine-forming bacteria can grow and produce histamine over a wide temperature range. The formation of histamine is more rapid, however, at high-abuse temperatures (e.g. 21.1°C or higher) than at moderate-abuse temperatures (e.g. 7.2°C). Once the enzyme histidine decarboxylase is present in the fish, it can continue to produce histamine in the fish even if the

bacteria are not active. The enzyme can be active at or near refrigeration temperatures. The enzyme remains stable while in the frozen state and may be reactivated very rapidly after thawing.

Symptoms of histamine poisoning

Symptoms of scombrotoxin poisoning include tingling or burning in or around the mouth or throat; rash or hives on the upper body; drop in blood pressure; headache; dizziness; itching of the skin; nausea; vomiting; diarrhea; asthmatic-like constriction of the air passage; heart palpitation; and respiratory distress. Symptoms usually occur within a few minutes to a few hours of consumption and last from 12 hours to a few days.

Source of histamine forming bacteria

The kinds of bacteria that are associated with histamine development are commonly present in the saltwater environment. They naturally exist on the gills, on external surfaces, and in the gut of live, saltwater fish, with no harm to the fish. Upon death, the defense mechanisms of the fish no longer inhibit bacterial growth in the muscle tissue, and histamine-forming bacteria may start to grow, resulting in the production of histamine. Evisceration and removal of the gills may reduce, but not eliminate, the number of histamine forming bacteria. The potential for histamine formation is increased when the scombrotoxin-forming fish muscle is in direct contact with the enzyme-forming bacteria.

Histamine formation during the processing of fish

The direct contact of fish with enzyme-forming bacteria occurs when the fish are processed (e.g. filleting) and can be particularly problematic when the surface-to volume ratio of the exposed fish muscle is large, such as in minced tuna for salads. Even when such products are prepared from canned or pouch retorted fish, recontamination can occur during salad preparation, especially with the addition of raw ingredients. The mixing in of the bacteria throughout the product and the high surface-to volume ratio can result in substantial histamine formation if time and temperature abuse occurs. At least some of the histamine-forming bacteria are halotolerant (salt tolerant) or halophilic (salt loving). Some are more capable of producing histamine at elevated acidity (low pH). As a result, histamine formation is possible during processes such as brining, salting, smoking, drying, fermenting, and pickling until the product is fully shelf-stable. Refrigeration can be used to inhibit histamine formation during these processes. A number of the histamine-forming bacteria are facultative anaerobes that can grow in reduced oxygen environments. As a result, reduced oxygen packaging (e.g., vacuum

packaging, modified atmosphere packaging, and controlled atmosphere packaging) should not be viewed as inhibitory to histamine formation. Histamine is water soluble (dissolves in water) and would not be expected in significant quantity in products such as fish oil that do not have a water component. However, histamine could be present in products such as fish protein concentrate that are prepared from the muscle or aqueous (water-based) components of fish tissue.

Fish species having potential to cause histamine poisoning

Sl. No.	Family	Scientific Name	Common Name
1.	Carangidae	*Alectis indica*	Indian Threadfish
		Alepes spp.	Scad
		Atropus atropos	Cleftbelly trevally
		Carangoides bartholomaei	Yellow Jack
		Carangoides spp.	Trevally
		Caranx crysos	Blue runner
		Caranx spp.	Jack/Trevally
		Decapterus koheru	Koheru
		Decapterus russelli	Indian scad
		Decapterus spp.	Scad
		Elagatis bipinnulata	Rainbow Runner
		Megalaspis cordyla	Horse Mackerel/Torpedo Scad
		Nematistius pectoralis	Roosterfish
		Oligoplites saurus	Leather Jacket
		Pseudocaranx dentex	White trevally
		Scomberoides commersonnianus	Talang queenfish
		Scomberoides spp.	Leather Jacket/Queen Fish
		Selene spp.	Moonfish
		Seriola dumerili	Greater/Japanese Amberjack or Rudder Fish
		Seriola lalandi	Yellowtail Amberjack
		Seriola quinqueradiata	Japanese Amberjack
		Seriola rivoliana	Longfin Yellowtail
		Seriola spp.	Amberjack or Yellowtail
		Trachurus capensis	Cape Horse Mackerel
		Trachurus japonicas	Japanese Jack Mackerel
		Trachurus murphyi	Chilean Jack Mackerel
		Trachurus novaezelandiae	Yellowtail Horse Mackerel
		Trachurus spp.	Jack Mackerel/Horse Mackerel
		Trachurus trachurus	Atlantic Horse Mackerel
		Uraspis secunda	Cottonmouth jack
2.	Chanidae	*Chanos chanos*	Milkfish
3.	Clupeidae	*Alosa pseudoharengus*	Alewife
		Alosa spp.	Herring
		Amblygaster sirm	Spotted Sardinella
		Anodontostoma chacunda	Chacunda gizzard shad
		Brevoortia patronus	Gulf Menhaden

		Brevoortia spp.	Menhaden
		Brevoortia tyrannus	Atlantic Menhaden
		Clupea bentincki	Araucanian herring
		Clupea harengus	Atlantic herring
		Clupea pallasii pallasii	Pacific herring
		Clupea spp.	Pichard/Shad/Herring
		Dorosoma spp.	Gizaard Shad
		Ethmalosa fimbriata	Bonga Shad
		Ethmidium maculatum	Pacific Menhaden
		Etrumeus sadina	Red-eye round herring
		Harengula spp.	Sprat/Herring
		Harengula thrissina	Pacific flatiron herring
		Hilsa spp.	Shad
		Nematolosa spp.	Gizzard Shad
		Opisthonema libertate	Pacific thread herring
		Opisthonema spp	Thread Herring
		Opisthopterus tardoore	Tardoore
		Sardina pilchardus	European Pilchard
		Sardinella aurita	Round Sardinella
		Sardinella gibbosa	Gold stripe Sardinella
		Sardinella longiceps	Indian Oil Sardine
		Sardinella maderensis	Madeiran Sardinella
		Sardinella spp.	Sardine
		Sardinops sagax	South American Pilchard
		Sardinops spp.	South American Pilchard
		Spratelloides gracilis	Silver-stripe round herring
		Tenualosa ilisha	Hilsa shad
		Tenualosa spp.	Shad
4	Coryphaenidae	*Coryphaena hippurus*	Mahi-Mahi /Dolphin fish
5	Engraulidae	*Anchoa* spp.	Anchovy
		Anchoviella spp.	Anchovy
		Cetengraulis mysticetus	Pacific anchoveta
		Engraulis capensis	Southern African anchovy
		Engraulis encrasicolus	European anchovy
		Engraulis japonicus	Japanese anchovy
		Engraulis ringens	Peruvian anchovy
		Engraulis spp.	Anchovy
		Stolephorus spp.	Anchovy
6	Istiophoridae	*Istiompax indica*	Black Marlin
		Istiophorus albicans	Atlantic sailfish
		Istiophorus platypterus	Indo-Pacific sailfish
		Kajikia albida	Atlantic white marlin
		Kajikia audax	Striped Marlin
		Makaira mazara	Indo-Pacific blue marlin
		Makaira spp.	Marlin/Sailfish
		Tetrapturus spp.	Marlin/Spearfish
		Tetrapturus spp.	Spearfish
7	Mugilidae	*Mugil cephalus*	Flathead Grey Mullet
8	Pristigasteridae	*Ilisha* spp.	Ilisha/Pellona
		Pellona ditchella	Indian pellona

9	Scombridae	*Acanthocybium solandri*	Wahoo
		Auxis spp.	Bullet Tuna/Frigate Tuna
		Cybiosarda elegans	Leaping Bonito
		Euthynnus affinis	Little tuna or Kawakawa
		Euthynnus spp.	Bonito
		Gasterochisma melampus	Butterfly kingfish
		Grammatorcynus spp.	Short Mackerel
		Gymnosarda unicolor	Dogtooth tuna
		Katsuwonus pelamis	Skipjack Tuna
		Orcynopsis unicolor	Plain Bonito
		Rastrelliger brachysoma	Short Mackerel
		Rastrelliger kanagurta	Indian Mackerel
		Sarda spp	Bonito
		Scomber australasicus	Blue mackerel
		Scomber japonicas	Chub mackerel
		Scomber scombrus	Atlantic mackerel
		Scomber spp.	Mackerel
		Scomberomorus cavalla	King Mackerel
		Scomberomorus commerson	Narrow-barred Spanish mackerel
		Scomberomorus guttatus	Indo-Pacific king mackerel/Spotted Spanish Mackerel
		Scomberomorus niphonius	Japanese Spanish mackerel
		Scomberomorus spp.	Spanish Mackerel
		Scomeromorus lineolatus	Streaked seerfish
		Thunnus alalunga	Albacore Tuna
		Thunnus albacares	Yellowfin Tuna
		Thunnus atlanticus	Blackfin Tuna
		Thunnus maccoyi	Southern bluefin tuna
		Thunnus obesus	Bigeye Tuna
		Thunnus orientalis	Pacific bluefin tuna
		Thunnus spp.	Tuna
		Thunnus thynnus	Atlantic bluefin tuna
		Thunnus tonggol	Longtail Tuna
10	Xiphiidae	*Xiphias gladius*	Swordfish

Source: Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011

Limits of histamine level in fish and fishery products

S. No.	Product Category	Applicable to	Histamine Level
1.	Raw/Chilled/Frozen Finfish	Species with high amount of free histidine (Listed fish species with the potential to cause histamine fish poisoning)	n=9, c=2; m=100 mg/kg, M=200 mg/kg
2.	Thermally ProcessedFishery Products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
3.	Smoked fishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
4.	Fish Mince/Surimi and analogues		n=9, c=2; m=100 mg/kg, M=200 mg/kg
5.	Battered and breadedfishery products		n=9, c=2; m=100 mg/kg,M=200 mg/kg

6.	Other Ready to Eat fisheryproducts		n=9, c=2; m=100 mg/kg,M=200 mg/kg
7.	Other value added fisheryproducts		n=9, c=2; m=100 mg/kg,M=200 mg/kg
8.	Other fish based products		n=9, c=2; m=100 mg/kg,M=200 mg/kg
9.	Dried/ Salted and Driedfishery products		n=9, c=2; m=200 mg/kg, M=400 mg/kg
10.	Fermented Fishery products		n=9, c=2; m=200 mg/kg, M=400 mg/kg
11.	Fish Pickle		n=9, c=2; m=200 mg/kg, M=400 mg/kg

Source: Food Safety and Standards (Contaminants, Toxins and Residues) Regulations, 2011

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

Satisfactory, if the following requirements are fulfilled:

1. the mean value observed is ≤ m
2. a maximum of c/n values observed are between m and M
3. no values observed exceed the limit of M,

Unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.

Control of histamine formation in fish

Freezing may inactivate some of the enzyme forming bacteria. Both the enzyme and the bacteria can be inactivated by cooking. However, once histamine is produced, it cannot be eliminated by heat (including retorting) or freezing. After cooking, recontamination of the fish with the enzyme-producing bacteria is necessary for additional histamine to form. For these reasons, histamine development is more likely in raw, unfrozen fish but should not be discounted in other product forms of scombrotoxin-forming fish species. Rapid chilling of scombrotoxin-forming fish immediately after death is the most important element in any strategy for preventing the formation of scombrotoxin (histamine), especially for fish that are exposed to warm waters or air, and for tuna which generate heat in their tissues. Some recommendations are:

a. Fish exposed to air or water temperatures above 28°C should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than 6 hours from the time of death; or

b. Fish exposed to air and water temperatures of 28°C or less should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than 9 hours from the time of death; or
c. Fish that are gilled and gutted before chilling should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than 12 hours from the time of death; or
d. Fish that are harvested under conditions that expose dead fish to harvest waters of 18°C or less for 24 hours or less should be placed in ice, or in refrigerated seawater, ice slurry, or brine of 4°C or less, as soon as possible after harvest, but not more than the time limits listed above, with the time period starting when the fish leave the 18°C or less environment.

Detection of histamine in fish

The most common method preferred for the determination of histamine is High Pressure Liquid Chromatography (HPLC). Chemical testing is an effective means of detecting the presence of histamine in fish muscles. However, the variability in histamine levels between fish and within an individual fish can be large. For this reason, a sampling plan has been provided by the FSSR of India for the analysis of histamine in fish. The number of samples 'n' (i.e., scombrotoxin forming fish) necessary to make a judgment about a lot depends on the anticipated variability, but should not be fewer than 09 samples per lot, unless the lot contains less than 09 fish, in which case a sample should be collected from each fish.

Principle

The sample is extracted by mixing with Perchloric acid. Precolumn derivatization is performed using dansyl chloride. The biogenic amines and the components in the solution are separated by HPLC with an appropriate column, using UV detection. Histamine mass concentration is calculated from the peak area ratio of histamine and internal standard with a calibration curve.

Reagents and Materials

- Acetone
- Acetonitrile
- Toluene
- Water, HPLC grade
- Water, distilled water, or equivalent
- Nitrogen gas
- Perchloric Acid

- Saturated Sodium Carbonate solution
- Dansyl Chloride Solution
- L- Proline
- Histamine stock solution
- Internal standard (IS) 1,7-diaminoheptane stock solution

Sample preparation

A pre-determined weight of the fish sample is taken and homogenized in perchloric acid in presence of 1,7-diaminoheptane. Then the sample is centrifuged for 5 min at 4°C. The supernatant is collected into a tube and mixed with sodium carbonate solution and Dansyl chloride solution. This solution is vortexed and incubated in the dark at 60 °C. After incubation, the tube is cooled and L-proline solution is added to it. The sample is vortexed and placed in the tube in dark for 15 min. Then toluene is added to this solution and kept in the freezer. The upper organic phase is transferred into a new tube and dried it in the fume hood with a stream of nitrogen. It is then re-suspended with acetonitrile/water (60/40 volume fraction) and vortexed. The solution is filtered in a glass autosampler vial and the autosampler is filled with the sample vails.

Fig 3. Ultra High Pressure Liquid Chromatography (UHPLC)

Results

The estimated concentration of histamine in the fish sample is expressed as mg/kg.

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Chapter 11

Introduction to HACCP Concepts

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Introduction

Safety of food remains a major concern in the seafood industry. The production and consumption of safe food are important to any society. The seafood safety is of more concerns in international fish trade due to its vast expansion recent decades. The export value of seafood had increased from US$8 billion in 1976 to US$ 160.5 billion in 2020 (FAO, 2021). The advent of emerging pathogens and the impacts of climate change on seafood safety major concern in fish processing industries. Each year, millions of illnesses can be attributed to contaminated food. Hence, a food safety system aimed at ensuring all food is as safe as possible is required. In this connection, the Hazard Analysis and Critical Control Points (HACCP) system is a single system that has been adopted by national and international bodies for ensuring seafood safety. However, HACCP system is not a standalone programme as it requires prerequisite programmes to work effectively. In present decade, the International Organization for Standardization (ISO) has developed the ISO 22000 family of standards on food safety management systems (FSMS) by taking approach of ISO 9001 as a management system, and incorporates the hygiene measures of prerequisite programmes and the HACCP principles and criteria.

The behaviour of consumers has been gradually changing. The consumer's awareness and demand of safe food is increasing every year. They currently require not only much higher dietary quality, hygiene and health standards in the products they purchase, but they also look for certification and reassurance of products' origins (national or geographical) and production methods. These change in customer's approach had led to adoption of HACCP system by the food processors in various countries to protect their customer's health. HACCP is a scientific and systematic approach to identify, assess and control hazards in the food production process. With the HACCP system, food safety control is integrated into the design of the process rather than relied on end-product testing. Therefore, HACCP system provides a preventive and thus cost-effective approach in food safety.

The HACCP system

HACCP system identifies, evaluates and controls hazards that are significant for food safety. HACCP system requires a team work. It requires firm commitment from top management level for effective

implementation. HACCP does not assure zero risk. It is a systematic tool to minimize risk of food safety hazards. HACCP plan once developed doesn't mean it is the ultimate plan. It needs to be modified whenever required. HACCP is a continuous process and is mainly risk based. HACCP need to be implemented from farm to fork. HACCP programme is a sum total of all pre- prerequisite programmes. The emphasis is on forecast rather than reaction, on getting the process right initially rather than correcting it after problems have occurred. It emphasized on identifying potential food safety problems and determining how and where these can be controlled or prevented. Describing what to do and training the personnel, implementation, recording and assurance throughout the food chain are taken care under HACCP system.

Pre-requisite programmes (PRPs)

PRPs such as standard operating procedures (SOP), sanitation standard operating procedures (SSOP), good manufacturing practises (GMP), etc. are implemented prior to HACCP plans. PRPs focus on employees, facilities and equipment and deals with illness policy, cleaning and sanitizing procedures, garbage removal, pest control, equipment selection, employee hygiene. It also deals with control of harvest operation and the overall plant environment which are not directly related to food (e.g. water quality, transportation and storage, plant sanitation, employee training, etc.).

Objectives of HACCP system

- Prevention of foodborne illness
- Reduction of economic losses due to product recall
- Protection of reputation
- Reduction of production costs
- To compete effectively in the international market

Benefits of HACCP system

- Increase food safety standards
- Increase food quality standards
- Ensures compliance with the regulatory guidelines and laws
- Promote teamwork
- Increase staff efficiency
- Due diligence defense in court

HACCP plan

It is a document prepared in accordance with the principles of HACCP to ensure control of hazards that are significant for food safety in the segment of the food chain under consideration. It is implemented following pre-requisite programmes. Prior to the application of HACCP to a fish or

seafood establishment, that establishment should be operating proper prerequisite programmes according to the Recommended International Code of Practice –General Principles of Food Hygiene (CAC/RCP 1-1969, Revision 2008/2020). Management awareness and commitment are necessary for the implementation of an effective HACCP system. The effectiveness will also rely upon management and employees having the appropriate HACCP knowledge and skills. Therefore, ongoing training is necessary for all levels of employees and managers, as appropriate. If the necessary expertise is not available on-site for the development and implementation of an effective HACCP plan, expert advice should be obtained from other sources, such as trade and industry associations, independent experts and regulatory authorities. Two steps are involved in HACCP plan preparation.

1. Conducts five preliminary steps
2. Applies the seven HACCP principles

Preliminary steps

- Step 1. Assemble the HACCP team.
- Step 2. Describe product.
- Step 3. Identify intended use.
- Step 4. Construct flow diagram.
- Step 5. Confirm flow diagram.

HACCP principles

- Principle 1. Conduct a hazard analysis and identify control measures
- Principle 2. Determine CCPs
- Principle 3. Establish validated critical limits
- Principle 4. Establish a system to monitor control of CCPs
- Principle 5. Establish the corrective actions to be taken when monitoring indicates a deviation from a critical limit at a CCP has occurred
- Principle 6. Validate the HACCP plan and then establish procedures for verification to confirm that the HACCP system is working as intended
- Principle 7. Establish documentation concerning all procedures and records appropriate to these principles and their application

HACCP plan is a final document that describes how a fish or seafood operation will manage the identified CCPs for each product under its particular environment and working conditions. The

following are the details on how to apply the above sequence for the preparation of a specific HACCP plan.

1. ***Assemble the HACCP Team***

HACCP Team consists of one HACCP coordinator with HACCP skills and other supporting members from various background. Larger companies - seven or eight people while small companies - two or three people. The HACCP coordinator should have responsibility for the whole HACCP program and be the Team leader.

The HACCP team should have access to all relevant and necessary information. The HACCP team should have expertise in the fields of management, production, quality assurance, maintenance, marketing and sales. The team should represent diverse personnel from the above fields.

2. ***Describe the product:***

A full description of the product should be drawn up, including relevant safety information such as: harvesting area and technique; raw materials and ingredients used including commercial and Latin name of the fish; factors that influence safety such as composition, physical/chemical parameters, such as water activity (aw), pH, salt content; processing such as heating, freezing, brining or smoking; packaging type; storage conditions and methods of distribution; shelf-life under specified condition should also be recorded.

3. Identify the intended use:

The intended use should be based on the expected uses by the end user or consumer. The use and preparation before use greatly influence the safety of the product. Certain products may carry harmful organisms as part of the natural flora. If the processing does not include a killing step, the only possibility to render the product safe is adequate heat treatment (e.g. cooking) during preparation. It is important to identify whether the product is to be used in a way that increases the risk of harm to the consumer, or whether the product is particularly used by consumers who are especially susceptible to a hazard. In specific cases, e.g. institutional feeding, vulnerable groups of the population, such as elderly and infants, must be considered.

4. Construct a process flow diagram:

A flow diagram should be constructed by the HACCP team to provide a clear and simple description of all steps involved in the operation. When applying HACCP to a given operation, consideration should be given to steps preceding and following the specific operation. Receiving and storage steps for raw materials and ingredients should be included. Time and temperature conditions during processing should be mentioned whenever there is a holding step, e.g. in holding vats, buffer tanks or other areas, where there could be a potential delay or temperature abuse.

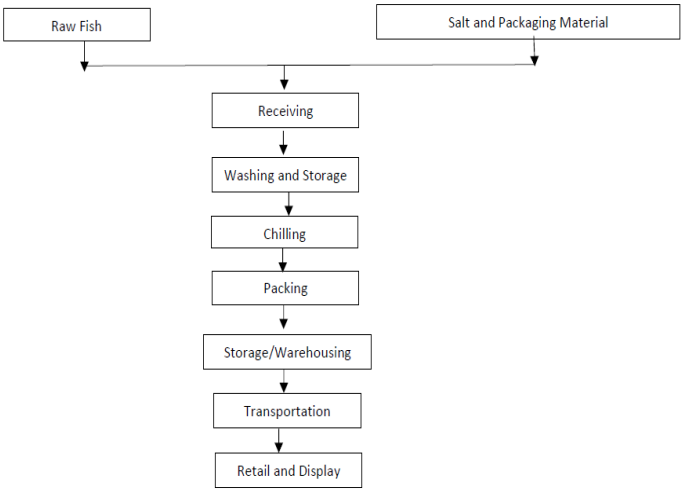

Fig 1. Example of flowchart of chilled fish

5. On site verification of the process flow diagram:

The HACCP team should confirm on-site the production operations against the flow diagram and amend it with information, such as correct durations, temperatures, and salt concentration, where appropriate. The site should be inspected during all hours (including night shifts and weekends) of operation to check for correctness and ensure that nothing crucial has been overlooked.

Principles of HACCP

1. Conduct a hazard analysis and identify control measures

A hazard is defined as a biological, chemical or physical agent in, or condition of, food (e.g. temperature abuse, insufficient thermal process), with the potential to cause an adverse health effect and harm. The HACCP team should list all hazards that may reasonably be expected to occur during production, processing, transportation and distribution until the point of fish consumption. Hazard analysis is the first HACCP principle and the science-based component of HACCP. An inaccurate hazard analysis would inevitably lead to the development of an inadequate HACCP plan. The HACCP team should identify which hazards are of such a nature that their elimination or reduction to acceptable levels is essential for the production of a safe product. A decision tree with a number of questions can be used to determine whether potential hazards are "real", as demonstrated below:

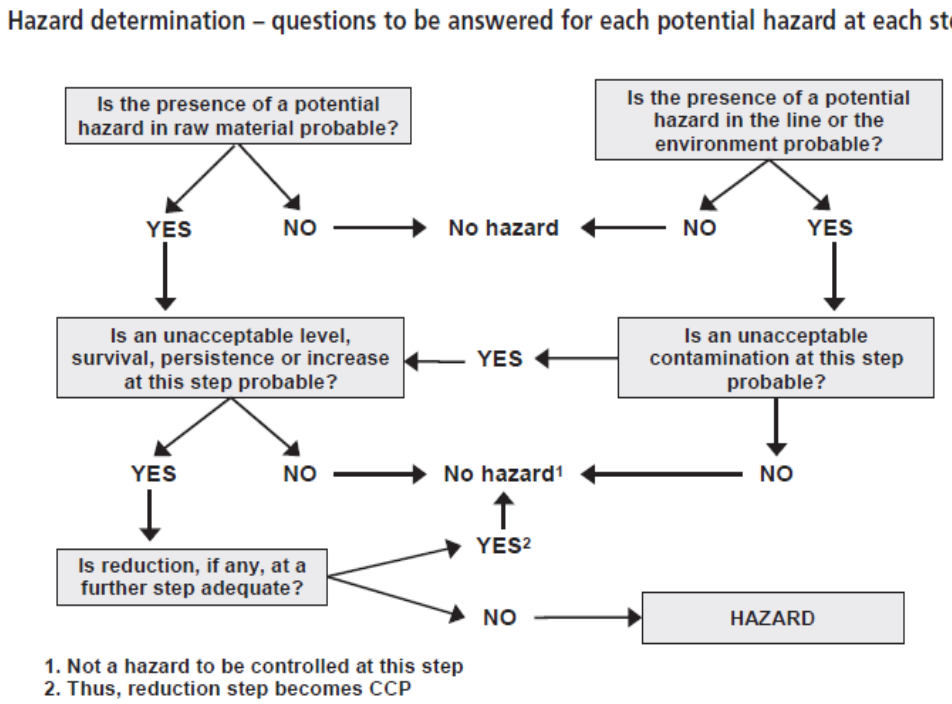

Fig 2. Hazard determination decision tree

Upon completion of the hazard analysis, the HACCP team must consider what control measures, if any, exist that can be applied for each hazard. More than one control measure may be required to control a specific hazard (or hazards) and more than one hazard may be controlled by a specific control measure. Control measures are activities that prevent, eliminate or reduce hazard to an acceptable level.

USFDA suggested following control measure for seafood-borne hazards:

Pathogenic bacteria:

- Time/temp control, heating/cooking, freezing, fermentation, salt/preservatives.

Pathogenic viruses:

- Cooking, source control from acceptable region

Parasites:

- Cooking, freezing.

Chemical hazard:

- Source control (Biotoxins, contaminants), time-temp (histamine), labelling (allergens)

Physical hazard:

- Source control (metal/glass), metal detector (metal pieces), PRPs

2. Determine CCPs

A CCP is a step at which control can be applied and is essential to prevent or eliminate a food safety hazard or reduce it to an acceptable level. CCPs are product and process specific. There may be more than one CCP at which control is applied to address the same hazard. Likewise, several hazards can be controlled at a single CCP. Complete and accurate identification of all the CCPs is fundamental for controlling food safety hazards. The determination of a CCP in the HACCP system can be facilitated by the application of a decision tree.

The application of the decision tree should be flexible depending upon the type of operation under consideration. Other approaches than the decision tree may be used for the determination of CCPs. If a hazard has been identified at a step where control is necessary for safety, and if no control measure exists at that step or at any other, then the product or the process should be modified at that step, or at an earlier or later stage, to include a control measure. This exercise should be conducted at each step and for each hazard to identify CCPs.

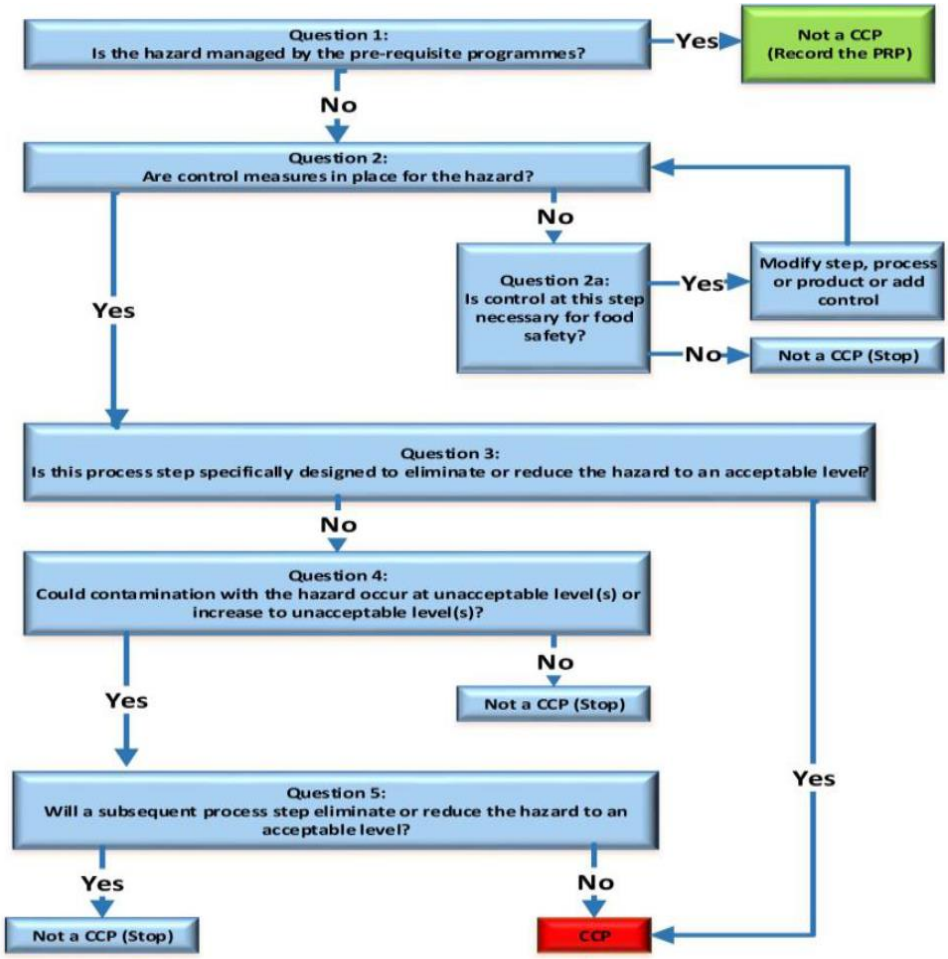

Fig 3. CCP decision tree

3. Establish validated critical limits

Critical limits are defined as criteria that separate acceptability from unacceptability. Critical limits represent the boundaries that are used to judge whether an operation is producing safe products as a result of proper application of the control measures. Critical limits should be scientifically based and refer to easily measurable factors such as temperature, time, chlorine levels, water activity (aw), pH, titratable acidity, salt concentration, available chlorine, preservatives, and sensory quality. Microbiological limits, which often require days for their measurement, should be avoided by all means. However, when microbiological limits are necessary, reliable rapid microbiological techniques should be used. The critical limits should meet the requirements of government regulations and/or company standards and/or be supported by other scientific data. It is essential that the persons responsible for establishing critical limits have knowledge of the process and of the legal and commercial standards required for the products. Example: There is a cooking (80°C for 2.5 min) step in the process line to control biological hazard. Here predefined time and temperature is the CL.

4. Establish a system to monitor control of CCPs

Monitoring is defined as the act of conducting a planned sequence of observations or measurements of control parameters to assess whether a CCP is under control. The monitoring procedures will determine whether the control measures are being implemented properly and ensure that critical limits are not exceeded. The monitoring procedures must be able to detect loss of control at the CCP. It can be qualitative or quantitative. It can be continuous or non-continuous. It can be of sensory evaluation, physical measurement (pH, a_w, humidity), chemical testing (chlorine level in water), microbiological examination (raw material and end product.

Components:

- What will be monitored?
- How the critical limit and control measures will be monitored?
- When (frequency)? and
- Who will monitor?

5. Establish the corrective actions to be taken when monitoring indicates a deviation from a critical limit at a CCP has occurred

As the main reason for implementing HACCP is to prevent problems from occurring, corrective actions should be predefined and taken when the results of monitoring at the CCP indicate a loss of control. Loss of control can cause a deviation from a critical limit for a CCP. All deviations must be controlled by taking predetermined actions to control the non-compliant product and to correct the cause of non-compliance. Product control includes proper identification, control and disposition of

the affected product. The establishment should have effective procedures in place to identify, isolate (separate), mark clearly and control all products produced during the deviation period. Corrective action procedures are necessary to determine the cause of the problem, take action to prevent recurrence and follow up with monitoring and reassessment to ensure that the action taken is effective. Reassessment of the hazard analysis or modification of the HACCP plan may be necessary to eliminate further recurrence. The control and disposition of the affected product and the corrective actions taken must be recorded and filed. Records should be available to demonstrate the control of products affected by the deviation and the corrective action taken. Adequate records permit verification that the establishment has deviations under control and has taken corrective action.

6. Validate the HACCP plan and then establish procedures for verification to confirm that the HACCP system is working as intended

Verification is the application of methods, procedures and tests, including random sampling and analysis and other evaluations, in addition to monitoring, to determine compliance with the HACCP plan. The objective of verification procedures is to determine whether the HACCP system is working effectively. Careful preparation and implementation of the HACCP plan does not guarantee the plan's effectiveness. Verification procedures are necessary to assess the effectiveness of the plan and to confirm that the HACCP system adheres to the plan. Verification should be undertaken by an appropriately qualified individual (or individuals) capable of detecting deficiencies in the plan or its implementation. Verification activities should be documented in the HACCP plan. Records should be made of the results of all verification activities. Records should include methods, date, individuals and/or organizations responsible, results or findings and actions taken. Apart from the initial validation, subsequent validation as well as verification must take place whenever there is a change in raw materials, product formulation, processing procedures, consumer and handling practices, new information on hazards and their control, consumer complaints, recurring deviations or any other indication, that the system is not working.

7. Establish documentation concerning all procedures and records appropriate to these principles and their application

Records and documentation are essential for reviewing the adequacy of and adherence to the HACCP plan. Several types of records should be considered among those relevant in an HACCP programme:

- Support documentation, including validation records, for developing the HACCP plan;
- Records generated by the HACCP system: monitoring records of all CCPs;
- Deviation and corrective action records, verification/validation records;

- Documentation on methods and procedures used;
- Records of employee training programmes.

Records may be in different forms, e.g. processing charts, written procedures or records, and tables. They can be stored in paper or electronic forms, provided that assurance of record integrity is provided. It is imperative to maintain complete, current, properly filed and accurate records. Failure to document the control of a CCP or implementation of a corrective action would be a critical departure from the HACCP plan.

Example of HACCP implementation in battered and breaded fishery product

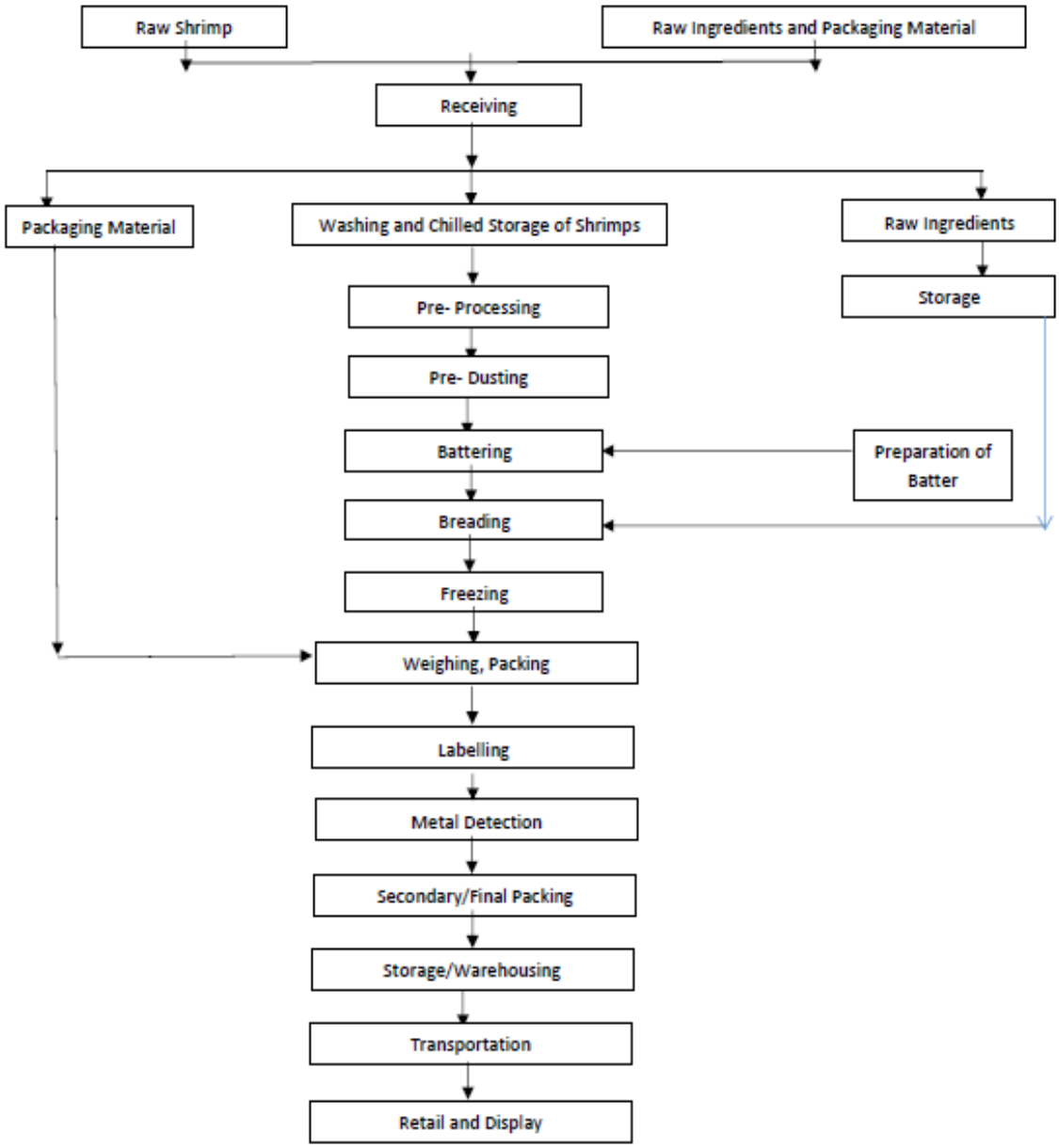

Fig 4. Example: IQF breaded shrimp

Conclusion

The safety of seafood products varies considerably and is influenced by a number of factors such as origin of the fish, microbiological ecology of the product, handling and processing practices and preparations before consumption. However, the food safety hazards and risk in seafood products cannot be made nil through any approach, it can only be minimized or reduced to an acceptable level. A large number of hazards are related to the pre-harvest situation or raw-material handling and must be under control by implementation of HACCP when the raw material is received at the processing factory.

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Chapter 12

Overview of chromatography and mass spectrometry

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Introduction

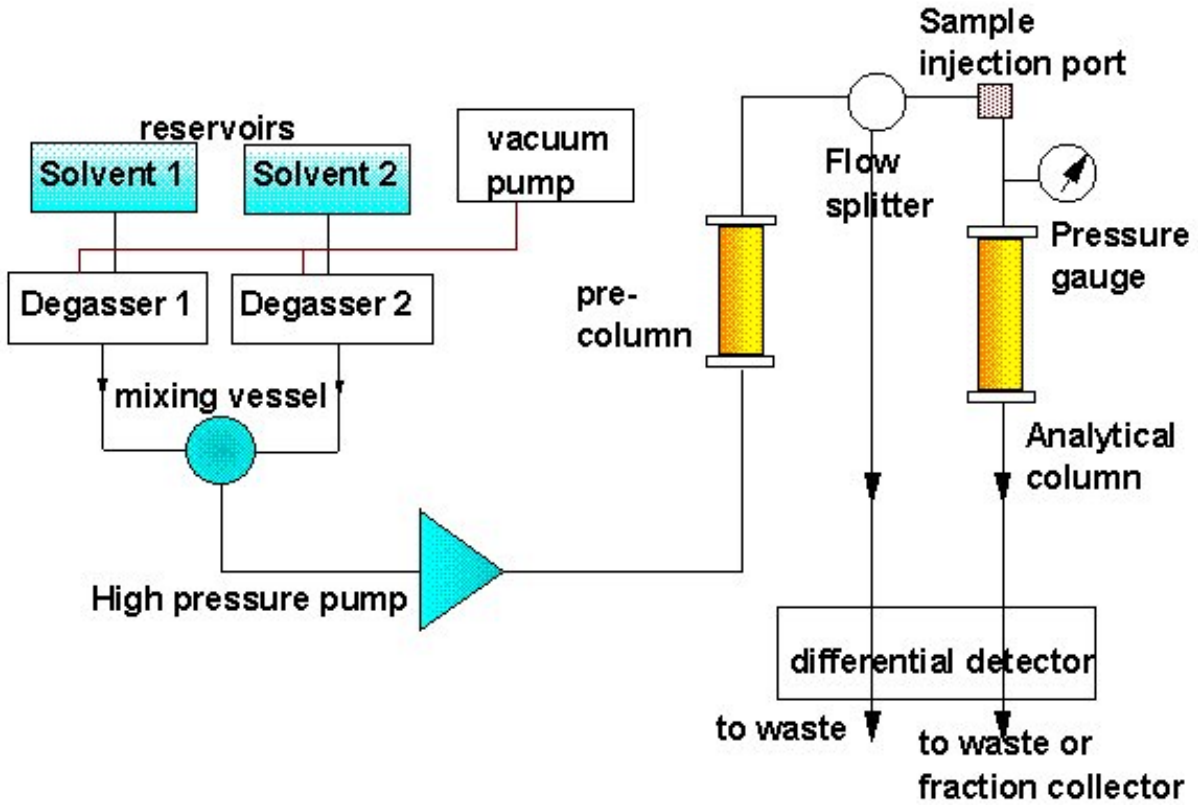
A Mass Spectrometer is an analytical instrument that measures the masses of individual molecules which have been converted into gas-phase ions. Molecules in a liquid-phase need to be converted into a gas-phase for the mass spectrometer to be able to measure them. Ions are separated, detected and measured by their mass-to-charge ratios (m/z). Mass spectrometers hyphenated with liquid chromatograph (LC-MS/MS) are widely used in chemical and biological research now a days, to identify and elucidate structures of unknown metabolites, protein etc. in biological tissue, plant material, microbial broth etc. It is also used for targeted analysis of known compounds such as pesticides, antibiotics, vitamins, amino acids, phospholipids etc. In the field of marine bioactive compounds, LC-MS/MS are widely used to determine metabolite profile of marine plants, fish, crustaceans, micro algae, and microbes. Structure elucidation of marine bioactive peptides is another possible application of LC-MS/MS. It is also used for targeted analysis of phenolic acids, flavonoids, carotenoids, vitamins, phospholipids etc. in the marine plants and animals.

Liquid Chromatography

A high-performance liquid chromatograph (HPLC) or ultra-high performance liquid chromatograph is a common front end of a LC-MS/MS system. HPLC/UHPLC separates mixture of compounds based on the principle of adsorption chromatography where the mobile phase is liquid solvent and the stationary phase is solid sorbent particles tightly packed inside a metal column. When the stationary phase is polar in nature, the type of chromatography is called normal phase chromatography; while in case of reverse phase chromatography the stationary phase is non polar. Reverse phase chromatography is most commonly used with mass spectrometry because of its repeatability, relatively lower maintenance, and chromatographic resolution for wide range of mid-polar to non-polar compounds. Most common type reverse phase stationary phase material is C18, where the silica particle surface is modified with 18 carbon chain length hydrocarbons. Similarly, C30 and C8 columns are used for separation of highly nonpolar and relatively polar compounds respectively. Normal phase

chromatography commonly uses unmodified silica as stationary phase and used for chromatographic separation of polar compound mixture such as fatty acids and tocopherol isomers. In reverse phase chromatography water in combination with acetonitrile or methanol is most common type of mobile phase, where the solvent elution programme starts with high aqueous content and gradually ramped to high organic content. In case of normal phase chromatography, water can not be used as mobile phase because of its interaction with silica particles. A combination of nonpolar and relatively polar solvents is used as mobile phase, where the elution programme starts with high content of nonpolar solvent and the content of polar solvent is gradually increased. The following figure presents different parts of the HPLC/UHPLC.

Figure. Different parts of a liquid chromatograph



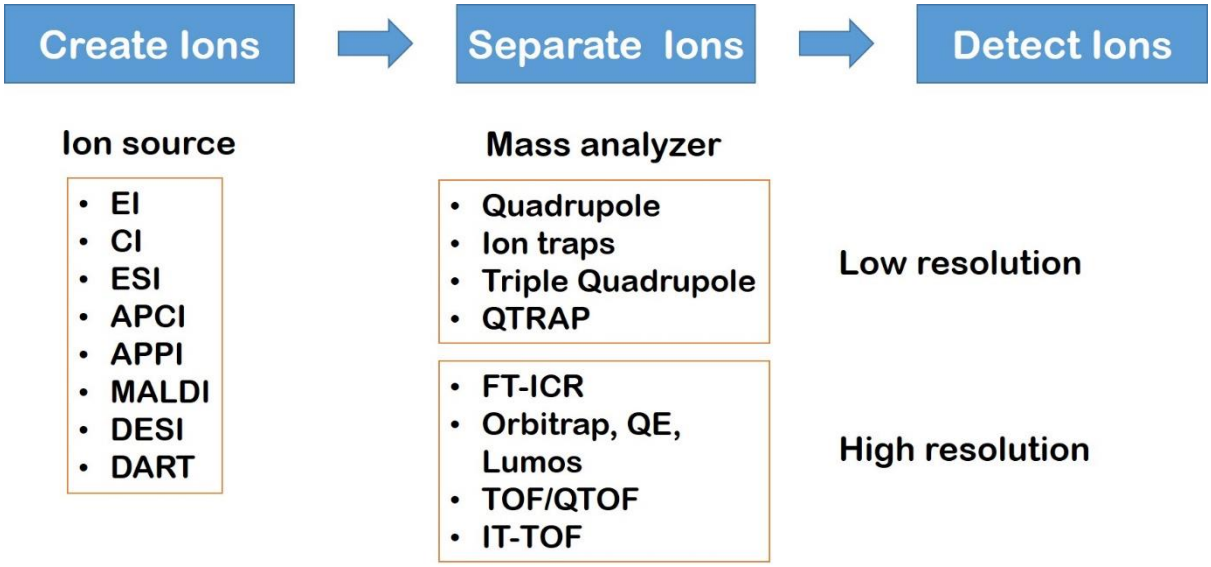
Chromatographic resolution is directly proportional to the length of the chromatographic column, and inversely proportional with the particle size, inner diameter, and pore size. Hence, a short length column with finer particle size, shorter inner diameter, and smaller pore size can achieve the same chromatographic resolution in less time which will take longer in a column of higher length, with bigger particle size, longer inner dia and bigger pore size. However, the solvent back pressure is extremely high in such short columns and can be used only with UHPLC where the pump is equipped to handle back pressure up to 18000 psi. UHPLC is a

popular front end of mass spectrometer due to short analysis time, sharp peak shape, and less consumption of organic solvents.

Mass spectrometer

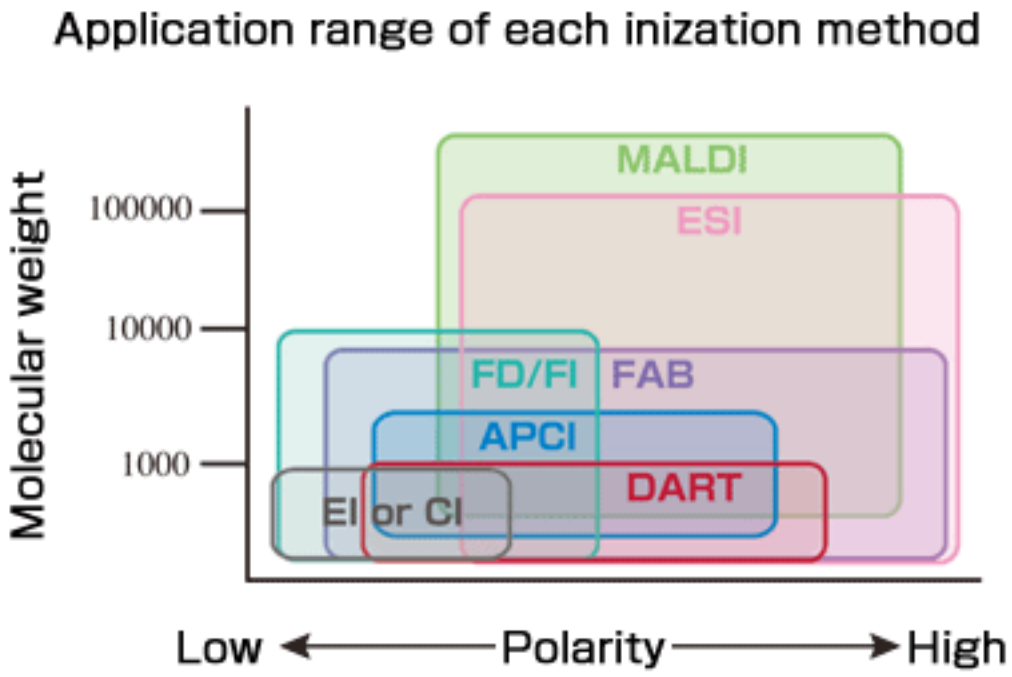
In a mass spectrometer the compounds introduced in liquid phase form gas phase ions in the ion source. Next the ions are separated in a mass analyzer and finally they reach the detector. The detector shows the output in the data system as a mass spectrum, total ion chromatogram (TIC), base peak ion (BPI) chromatogram, or extracted ion chromatogram (XIC). There are different possible ion sources and mass analyzer combinations in different mass spectrometers which are used for different application needs. The following figure shows a schematic of major parts of a mass spectrometer and lists different possible ion sources and mass analysers.

Figure. Schematics of different parts of mass spectrometer



The electron impact (EI) and chemical ionization (CI) ion sources are found in gas chromatograph hyphenated mass spectrometer (GC-MS) and the ionization happens under complete vacuum. EI is a hard ionisation technique, where the molecular weight ion is almost completely broken down into fragments. Hence, for molecular weight determination, CI ion source is preferred in GC-MS; where a pseudo molecular ion with reagent gas (most commonly methane or ammonia) is formed through a soft ionisation technique. Electron spray ionisation (ESI), atmospheric pressure chemical ionisation (APCI), atmospheric pressure photo ionisation (APPI), fast atom bombardment (FAB), matrix assisted laser desorption ionisation (MALDI), desorption electron spray ionisation (DESI), direct analysis in real time (DART) are prominent ion sources in different LC-MS. These ion sources are used based on the polarity and molecular weight range of the target analytes or analyte classes, as shown in the following schematics.

Figure. Application range of different ion sources



ESI is most commonly used ion source with LC-MS as a wide range of compounds with medium polarity to high polarity, and low to high molecular weight can be analysed. APCI is suitable for compounds with low polarity which do not ionise sufficiently in ESI. APPI is suitable for highly non polar compounds such as persistent organic pollutants. MALDI is prominently used for intact mass determination of proteins.

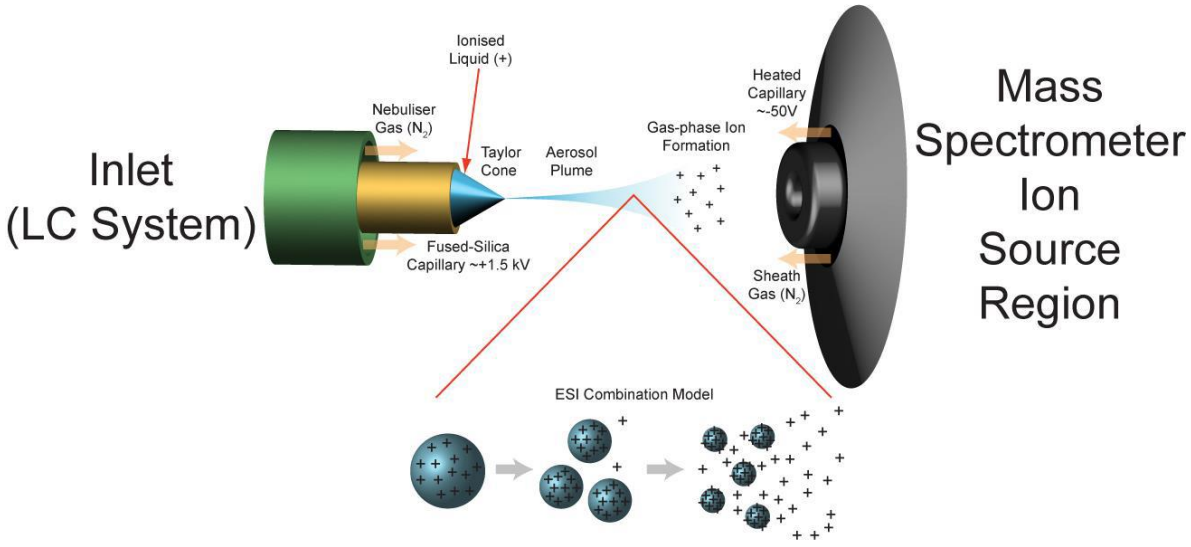
In ESI ion source, the compound in liquid phase is nebulised through a charged capillary. In positive ionisation mode a positive charge is applied, where in negative ionisation mode a negative charge is applied. The solvent around the droplets containing charged ions is rapidly evaporated by the heater gas and ion source temperature. Hence, the droplets become smaller and smaller, finally releasing only gas phase ions. The ESI is a soft ionisation technique, where the most commonly formed ions are $[M + H]^+$, and $[M - H]^-$, depending on the ionisation operating mode. These ions of a compound are called adduct/pseudo molecular weight ion/parent ion/precursor ion. Some other common adducts found in ESI ion source are listed below.

Positive polarity adduct	Mass difference*	Negative polarity adduct	Mass difference*
$[M + H]^+$	+1.0078	$[M - H]^-$	-1.0078
$[M + NH_4]^+$	+18.0344	$[2M - H]^-$	-
$[M + Na]^+$	+22.9898	$[M - H + H_2O]^-$	+18.0106
$[M + K]^+$	+38.9637	$[M - H + CH_3OH]^-$	+32.0262
$[M + H_2O + H]^+$	+18.0106	$[M - H + CH_3CN]^-$	+41.0265
$[M - H_2O + H]^+$	-17.0027	$[M + Cl]^-$	+36.4609
$[M - 2H_2O + H]^+$	-35.0133	$[M + Br]^-$	+79.9040

A cone voltage or declustering potential is applied on the ion source cone to further push the generated gas phase ions towards the mass analyser. Hence, the flow rate of nebuliser gas,

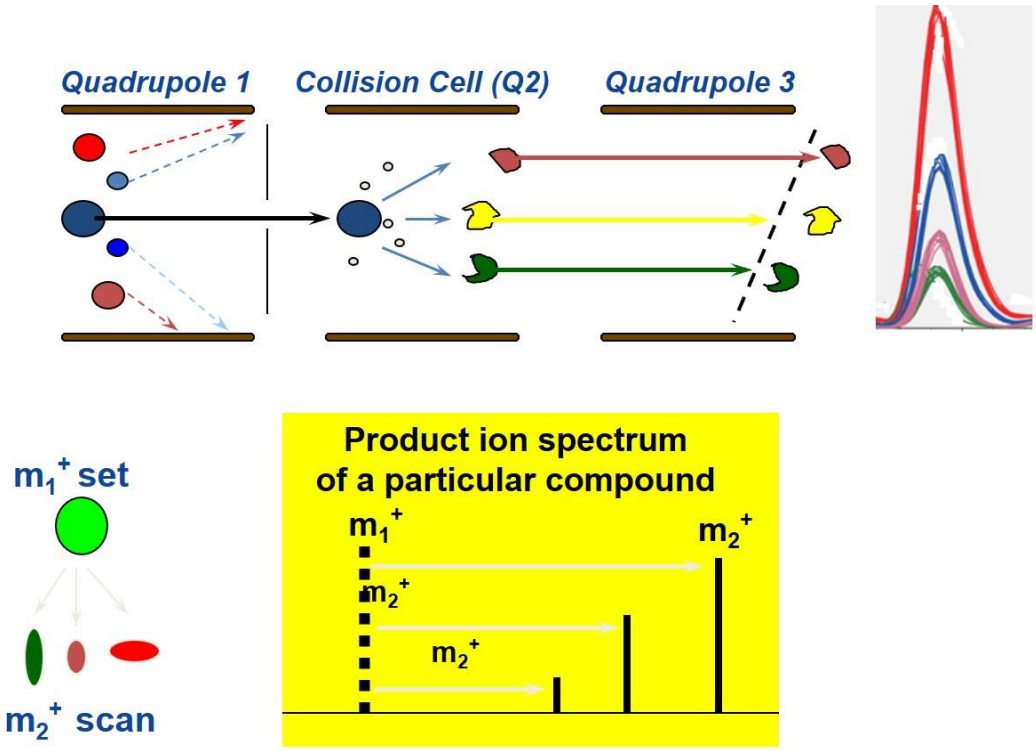
heater gas, ion source temperature, cone voltage/declustering potential are important parameters that need to be optimised in analyses using ESI ion source. Following is a schematic of the operation of ESI ion source.

Figure. Schematic of ESI ion source.



A quadrupole system uses four cylindrical magnets that are set parallel to each other and function to filter ions based on their mass-to-charge ratio (m/z). The analyzer consists of two pairs of like charged magnets that oppose each other and keep the ions within the ion path of the quadrupole under vacuum. Ions are filtered based on their masses as they traverse the linear ion path. When a linear series of three quadrupoles is used, the resulting triple stage quadrupole analyzer is able to both filter and fragment the ion stream. In most cases, the first (Q1) and third (Q3) quadrupoles act as mass filters, while the second (Q2) quadrupole dissociates ions by having them collide with argon, helium or nitrogen gas. Quadrupole-based mass analysers excel at tracking single ions or reactions for extended periods of time. This is why they are preferentially used in the targeted analysis of compounds, especially known compounds such as drugs and pollutants. This is also why quadrupole mass analysers are often used in the fields of food safety, environmental analysis, clinical and forensic toxicology studies. The triple quadrupole (QQQ) mass spectrometer (MS) consists of a series of three quadrupoles and selects ions of specific mass-to-charge ratios (m/z) when a specific DC/RF voltage combination is applied. The first and third quadrupoles (Q1) act as mass filters, while the Q2 acts as a collision cell. Triple quadrupole MS systems can be operated in a tandem MS/MS assay called Selected Reaction Monitoring (SRM) (sometimes also called Multiple Reaction Monitoring (MRM)) mode. SRM is a highly selective mode whereby a fixed set of DC and RF voltages is applied

to the quadrupole, permitting only one precursor ion, which is measured by its m/z, to pass. After the Q1 filters that specific precursor ion, the Q2 produces product ions via collision of the precursor ion with a neutral gas (e.g., nitrogen) in a process called collision-induced dissociation (CID). Product ions progress to the Q3, where only a specific m/z is permitted to pass. By breaking the ion apart into its component fragments, a given molecular species can be identified not only by its mass but by product identity. In this way, SRM reduces noise and increases selectivity. Following schematic presents the working of a triple quadrupole mass analyser in MRM mode.



LC-MS/MS is a versatile technology with wide range of application in marine bioactive compound analysis. LC-MS/MS can be used for free amino acid analysis in serum, tissue or plant material extracts. The instrument with ESI and MALDI ion source has prominent application in the field of proteomics and peptide sequencing of bioactive peptides. The technique is also used for structural elucidation of bioactive compounds through molecular weight determination, and tandem mass spectra fragmentation pattern. High resolution mass spectrometer can be used for high throughput metabolomic profile of biological materials and can derive important insights in biological experiments.

Chapter 13

Analysis of Pesticide Residue in Fish

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Pesticides are chemical compounds that are used to kill pests, including insects, rodents, fungi, and unwanted plants (weeds). Over 1000 different pesticides are used around the world. Pesticides are used in public health to kill vectors of disease, such as mosquitoes, and in agriculture to kill pests that damage crops.

Pesticide can be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest such as insect, rodent, nematode, fungus, weed, other forms of terrestrial or aquatic plant or animal life or viruses, bacteria, or other microorganisms on or in living man or other animals, which declares to be a pest, and any substance or mixture of substances intended for use as a plant regulator, defoliant, or desiccant.

Pesticides can be classified in many ways on the basis of use, toxicity, mode of entry, mode of action, chemistry, and formulations. The major chemical types of pesticides include

(i) **Organochlorine pesticides (OC)** – This group consists of the polychlorinated derivatives of cyclohexane (Lindane), polychlorinated biphenyls (DDT, dicofol), and polychlorinated cyclodiene (Endosulfan).

Properties of OCs

Physical property: OCs are solids that possess low volatility, low solubility in water, high solubility in oils, fats, lipids, *etc*., and they are not prone to environmental degradation.

Chemical property: Organochlorine pesticides shows isomerism

Toxicity: These compounds possess high acute toxicity as well as chronic toxicity

Biological stability: OCs are not rapidly degraded by enzymes, not rapidly exerted, but get stored in the fatty tissues.

A number of organochlorine pesticides have been banned globally and they are controlled via the Stockholm convention on persistent organic pollutants (POP's). These include: aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, and toxaphene

(ii) **Carbamates**: Carbamates are esters of either carbamic acids or thiocarbamic acids. Carbamates may be further subdivided into three sub-groups,

Group	Example
Aryl N methyl carbamate	Carbaryl, Propoxur
Hetero cyclic mono or dimethyl Carbamates	carbofuran
carbamoylated oximes	methomyl
Thiocarbamates	cartap hydrochloride (neriestoxin group of insecticide)

Properties:

Physical property: The organo-carbamates are available as nonvolatile solids. Carbaryl, and carbofuran are having very low water solubility (40-6000ppm) whereas Cartap hydrochloride is hygroscopic in nature. And these compounds undergo degradation by environmental factors.

Chemical property: These compounds are unstable in an alkaline medium.

Toxicity: The organo-carbamate compounds exhibit moderate to extreme toxicity, and they do not display chronic toxicity

Biological stability: The organo-carbamate compounds undergo enzymatic degradation and are rapidly metabolized and excreted. Biomagnification is almost absent in this group of pesticides and chronic toxicity is insignificant.

(iii) **Organophosphates (OP):** These are the esters of derivatized phosphoric acid, thiophosphoric acid and dithio phosphoric acids which are called phosphates, thiophosphates and dithiophosphates respectively. Some of the examples of each class of pesticides are as follows:

Group	Example
Phosphates	monocrotophos,phosphamidon, 2,2-dichlorovinyl dimethyl phosphate (DDVP) or Dichlorvos
Thiophosphates	methyl parathion, fenitrothion, Phosphorothiates oxy demeton methyl
Dithiophosphates	phosporodithioates dimethoate, pphosphorothioates

Based on the organic moiety attached to the phosphoric acid these can also be classified into aliphatic, phenyl, and heterocyclic derivatives.

Properties

Physical property: These compounds are available as liquids or semi-solids and possess significant vapour pressure and are comparatively volatile in nature. Some of these compounds are slightly soluble in water (example: Phosphamidon).

Chemical property: These compounds which are esters of phosphoric acid are not stable in alkaline pH, but stable over a narrow range of pH. Thiophosphates and dithiophosphates

undergo molecular rearrangements, forms isomers with increased toxicity and undergo oxidation to give oxo compounds with increased toxicity. The organo phosphorous pesticides undergo the conversion of one pesticide into another pesticide. The following are some examples.

Trichlorfon → dichlorvos

Formothion → dimethoate

Acephate → methamidophos

Toxicity: These compounds exhibit acute extreme toxicity to slight toxicity. LD50 values may change with the purity of the compound. These compounds are having low chronic toxicity. They undergo rapid conversion into low fat-soluble metabolites which are excreted.

Biological stability: The OP compounds undergo enzymatic degradation and the metabolites are fat-soluble and easily get excreted. Biomagnification is almost absent and chronic toxicity is insignificant

(iv) **Pyrethroids** – Living organisms do contain naturally a large number of chemicals some of which give them protection from foreign invasive substances. Many such chemicals have been isolated, identified, and evaluated for their biological activity. The flowers of chrysanthemum contain compounds called pyrethrins which are found to have possessed very good pesticidal activity but are found to be less stable in the environment. The pyrethrins are chemically the esters of chrysanthemic acid and pyrethric acid (which contains dimethyl cyclopropane group) with alcohols, namely pyrethrolone, cinerolone and jasmolone.

Synthetic Pyrethroids: Allethrin was the first synthetic pyrethroid developed in 1949, followed by resemethrin. However, they have failed to contain the desired properties and proved to be highly photolabile. The first photo sable pyrethroid developed was permethrin. This was followed by cypermethrin, deltamethrin, and fenvalerate. The synthetic Pyrethroids contain a halogenated derivative of dimethyl cyclopropane carboxylic acid and cyano phenoxy benzyl alcohol. Fenvalerate is an exception with the acid portion being p-chlorophenyl isopropyl acetic acid instead of cyclopropane carboxylic acid. In the case of permethrin, the alcohol portion does not have cyano - group, but it is simply phenoxy benzyl alcohol.

Water bodies around the world are threatened by various anthropogenic activities, resulting in poor water quality. The pesticide contamination in fish mainly comes through agricultural runoff and municipal sewage effluent. Later, intensive aquaculture practices with insecticides such as trichlorfon and dichlorvos to kill unwanted organisms or as algaecides to control water quality in fish/shrimp farms of different regions of the world also lead to pesticide

contamination in fish. Several studies have reported the presence of organochlorine pesticides in fish harvested from Indian waters also.

Procedure for Pesticide residue analysis in fish by GC-MS/MS

- Take a 2 kg fish sample, and homogenize the muscle tissue. Weigh 5 g representative sample in 50 ml centrifuge tube
- *Extraction*: Add 5 ml distilled water and Vortex for 1 minute. Then add 10 mL of acetonitrile containing 100 μL of acetic acid and vortexed for 1 minute. Add 6 g of magnesium sulphate ($MgSO_4$) and 1.5 g of sodium acetate (CH_3COONa) and vortex for 2 minutes. Centrifuge the content at 4000 rpm for 5 minutes. Keep the supernatant at -20°C for 20 minutes n to avoid loss of thermo-labile analytes due to heat generated during dSPE clean-up
- *Clean up*: Transfer the extract to dispersive-SPE (dSPE) tubes containing 50 mg PSA sorbent + 150 mg MgSO4 per mL as per AOAC- QuEchERS 2007.01. Vortex the mixture for 1 minute. Centrifuge at 4000 rpm for 5 minutes
- Filter 2ml extract through 0.2µ PTFE membrane filter; Inject and analyse in GC-MS

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Chapter 14

Seafood Preservation and Value Addition

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Seafoods play a major role in satisfying the demands of the diverse global customers on account of its availability, accessibility, nutritional as well as quality and safety aspects. These valuable commodities are simultaneously convenient due to its potentiality for high product diversification. Though an excellent source of nutrient with quality protein, omega-3 fatty acids, essential vitamins and minerals, it is highly perishable too, which demands its effective processing and preservation. Effective approaches in this regard is crucial to maintain its inherent quality characteristics and extended stability for better returns, to abate post-harvest losses as well as for consumer safety and satisfaction. Preservation aims to delay, lessen or hinder spoilage on account of chemical, enzymatic or microbial causes which can be achieved by controlling the storage temperature, maintaining proper water activity, proper pH, use of preservatives, alone or in combination. Among the various preservation methods available, low temperature preservation viz., chilling as well as freezing has attracted interest of many researchers on account of its minimal changes in the texture and other characteristics of fish if properly processed and stored. Traditional preservation techniques, collectively known as curing techniques viz., drying, salting, smoking, pickling etc. are commonly adopted methods for seafood preservation. The demand for fishery products with minimal processing has drawn the attention of innovative processing and preservation technologies like retort processing, high pressure processing, irradiation, pulse light technology, cold plasma processing, ohmic heating etc. to preserve and extend the quality and stability of these valuable commodities without compromising its nutritional, functional, and sensory characteristics.

Value addition approaches

Being one of the fastest growing economies and the second largest consumer market in the world, India offers a strong platform for processed seafood industry. As far as fish processing industry is concerned value addition is one of the possible approaches to raise profitability on account of the highly competitive and increasingly expensive nature of the industry. In foods, value is a combination of functional attributes as well as emotional benefits arising on account of nutritional as well as sensory facets at superior quality as well as affordable price. In

addition, it promises utilization of the under-exploited nutrient rich resources in the most effective manner. Value can be added to fish and fishery products according to the requirements of different markets. Value added fish products are presented in a preparatory and convenient form such as dressed/trimmed, minced etc. or those that have added ingredients such as a coating, bioactive/functional constituents in it. A number of such diverse products have already invaded the industry, globally ranging from live fish and shellfish to ready to serve convenience products. Value added fishery products primarily fall under the categories viz., mince/mince-based products, surimi/surimi-based products, enrobed or coated products, ready to serve retorted products, cold/hot extruded products, speciality products, ethnic products like marinated, dried products etc.

Fish mince can be defined as deboned and unwashed fish flesh from fillets or frames and is produced at the initial step of surimi manufacturing. When compared to surimi, fish mince can be obtained at a significantly higher yield with much less capital investment. Fish mince also offers nutritional advantages, economic benefits as well as functional advantages compared to the other intermediate materials. Fish mince can also be successfully used directly in various food systems and in a physically or chemically altered form to produce an array of nutritional and functional products. It finds application in processing several convenience foods like fish finger, cutlet, burger and also in some low cost salted and dried products. For preparation of fish finger, stick, etc., the mince stripped from the bone frame is incorporated to increase the yield.

Surimi is a Japanese term for mechanically deboned fish flesh that has been washed with water and mixed with cryoprotectants for good frozen shelf life. Washing not only removes fat and undesirable matters such as blood, pigments and odoriferous substances but also increases the concentration of myofibrillar protein, the content of which improves the gel strength and elasticity of the product. This property can be made use in developing a variety of products like fish sausage, balls, burgers as well as fabricated products like shellfish analogues which fetches good demand in both domestic and export markets. Low cost fishes can also be conveniently used for the preparation of surimi. Block frozen surimi and surimi-based products are popular, especially in South east Asian countries.

Coated/battered and breaded commodities are highly appreciated form of value-added products on account of their convenience, sensory appealness and nutritional attributes. In view of the increasing consumer demand, the technology has made several advancements. The most important advantage of coating is value addition as it increases the bulk of the product. This

technology also paves way for better utilization of underutilized seafood resources. A wide array of seafood products can be categorized in it, with the first commercially launched coated product being fish finger/fish stick followed by commodities in similar line viz., coated fish fillet, fish portions, fish cakes, fish medallions, fish nuggets, breaded oysters and scallops, crab balls, fish balls, coated shrimp products, coated squid rings etc. The most popular battered and battered products in India include fish nuggets, cutlet, balls, finger, patties etc. Various ready to eat novel battered and breaded snack products have good scope in value added markets.

Ready to serve fish products are gaining popularity in both domestic and export market. A wide array of products are categorized under this including retorted fish curries, rice-fish combos, seafood biriyanis etc. These products have a shelf life of more than one year at room temperature. The most common retortable pouch consists of a 3-ply laminated material consisting of polyester/aluminium/cast polypropylene. As there is increasing demand in domestic and International market for ready to serve products, proper exploration of this technology can provide a lively market for these commodities. The technology for retort pouch processing of several varieties of ready to serve fish and fish products including curries from mackerel, rohu, sardine, tuna, pomfret, prawn, seer fish molly, pearl spot molly, fried mussel, fish sausage, prawn kurma, prawn manchurian, fried mussel masala etc. has been standardized at ICAR-CIFT and this technology has been transferred successfully to entrepreneurs.

Food extrusion provides a great versatility for the development of low-cost, high-nutritive and convenient food products such as cereal-based snacks and food products. Extruded products are gaining importance nowadays on account of their unique flavour, texture and convenience. Extruded products contain low levels of protein, which makes it necessary to fortify them with protein-rich diets. One of the possible ways for alleviating this problem is to utilize fish and fish proteins to enrich cereal-based extruded products. Formulation of appropriate types of products using fish meat and fish portions will add value to the low-cost and underutilized fish and shellfish, thus promoting their utilization. Attractive packaging for the products and market studies are needed for the popularization of such products. These products can command very high market potential particularly among the urban elites.

Product diversification is becoming mandatory for effective marketing and currently, speciality products that are more convenient viz., ready to cook/eat are getting more consumer acceptance. The most popular products under the speciality product category include those like

stretched shrimp (Nobashi), sushi (Cooked butterfly shrimp), skewered shrimp, shrimp head-on cooked (centre peeled), fish wafers, fish crackers, fish soup powder etc.

Ethnic seafood products are those that are region specific and are being prepared and consumed by different people since ancient times. Some of these EFP are preserved or processed using centuries-old indigenous knowledge of fermentation/drying/smoking etc. Globalization has resulted in high demand for these ethnic food products and hence approaches towards its popularization by adopting various processing techniques can bring a huge market potential for these commodities.

Packaging

Packaging makes food more convenient and gives the food greater safety and stability by protection from microbial, chemical or biological changes. Different packaging techniques are employed as supplement to other preservation techniques to maintain the freshness and quality of seafood products. Of these, the advanced methods include vacuum packaging, modified atmosphere packaging, active packaging, intelligent packaging etc. Vacuum packaging involves the removal of air from the package and the application of a hermetic seal. The air removal creates a vacuum inside the packs and lack of oxygen in packages minimize the oxidative deteriorative reactions and aerobic bacterial growth. Vacuum packaging can considerably extend the viable shelf life of many cooked foods. The use of vacuum packaging, in gas impermeable and heat stable materials, has many advantages, which include; no or low risks of post pasteurisation contamination, ease of handling, inhibition of growth of aerobic spoilage organisms and inhibition or slowing of deleterious oxidative reactions in the food during storage due to oxygen barrier properties of the packaging material. Vacuum packed foods maintain their freshness and flavor longer than with conventional storage methods, as there is no contact with oxygen.

Modified atmosphere packaging, a technologically viable method has been developed as a complement to low temperature preservation like icing/chilling facilitating to reduce the quality losses and extend the storage life of fresh seafood products. In modified atmosphere packaging air is replaced with different gas mixtures to regulate microbial activity and /or retard discolouration of the products. The proportion of each component gas is fixed when the mixture is introduced into the package; however, no control is exercised during storage. The composition of the gas mixture changes from its initial composition as a result of chemical, enzymatic and microbial activity of the product during storage. It is primarily the enrichment of carbon dioxide in the storage atmosphere as a means of controlling microbial growth, which

results in the extension of shelf life of products. The gases normally employed are carbon dioxide, mixtures of carbon dioxide and nitrogen, carbon dioxide and oxygen and carbon dioxide, oxygen and nitrogen with the sole objective to extend the shelf life of the product beyond that obtained in conventional refrigerated storages. The composition of the gas mixtures used for MAP of fresh fish varies, depending upon whether the fish in the package is lean or oily fish.

Active packaging, an innovative approach in packaging changes the condition of the packaging and maintains these conditions throughout the storage period to extend shelf-life or to improve safety or sensory properties while maintaining the quality of packaged food. This is attained by incorporation of certain additives into the packaging film or within packaging containers with the aim of maintaining and extending product shelf life. Active packaging technique include the incorporation of either scavenging or emitting systems to emit and/or to remove gases during packaging, storage and distribution. Some of the active packaging systems include oxygen scavengers, carbon dioxide emitters, moisture regulators, antimicrobial packaging, antioxidant releasers, release or absorption of flavours and odours, carbon dioxide scavenger and active packaging systems with dual functionality etc.

Intelligent packaging, sometimes referred as smart packaging, senses some properties of the food it encloses or the environment in which it is kept and informs the manufacturer, retailer and consumer of the state of these properties. Intelligent packaging has been defined as 'packaging systems which monitor the condition of packaged foods to provide information about the quality of the packaged food during transport and storage. These include time temperature indicators, leakage indicator, freshness indicator, etc. Active and intelligent packaging systems contribute to the improvement of food safety and extend the shelf-life of the packaged foods.

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Practicals

Introduction to Basic Laboratory Concepts and Preparation of Reagents

Basic Concepts of Preparing Solutions

In the lab, it's important to prepare solutions with precise concentrations and amounts of various chemicals. It's important to measure and dispense the solute and solvent accurately to achieve the desired concentration. Common methods of dispensing solutes include weighing and pipetting, while dispensing solvents can be done using volumetric flasks, graduated cylinders, or pipettes. Properly preparing and handling solutions is essential to ensure accurate and reliable experimental results in laboratory work.

Preparing solutions is a fundamental task in laboratory work and involves mixing one or more solutes in a solvent to obtain a homogeneous mixture. The concentration of a solution is the amount of solute present in a given amount of solution. Solutions can be prepared by weighing or measuring the appropriate amounts of solutes and solvents and mixing them together. To prepare a solution with a specific concentration, it is necessary to know the amount of solute required to make the desired concentration and the volume of solvent needed. The amount of solute can be calculated using the equation:

amount of solute (in moles) = concentration (in M) x volume of solution (in L)

Concentration is a general measurement unit stating the amount of solute present in a known amount of solution.

$$\text{Concentration} = \frac{\text{amount of solute}}{\text{amount of solution}}$$

Percent solutions are a common way to express the concentration of a solution in a laboratory. A percent solution is the amount of solute per 100 units of solution, typically expressed as a percentage. There are several different ways to express percent solutions in a laboratory:

1. Weight/Weight Percent (w/w%): This is the weight of the solute divided by the weight of the solution, multiplied by 100%. For example, a 10% w/w solution of sodium chloride (NaCl) contains 10 grams of NaCl per 100 grams of solution.
2. Weight/Volume Percent (w/v%): This is the weight of the solute divided by the volume of the solution, multiplied by 100%. For example, a 5% w/v solution of glucose contains 5 grams of glucose per 100 milliliters of solution.
3. Volume/Volume Percent (v/v%): This is the volume of the solute divided by the volume of the solution, multiplied by 100%. For example, a 20% v/v solution of ethanol contains 20 milliliters of ethanol per 100 milliliters of solution.

Percent solutions can be useful when precise concentrations are not required, but a general understanding of concentration is necessary. They are often used in the preparation of common laboratory solutions, such as buffers and reagents. However, it's important to note that percent solutions are not as precise as other methods of expressing concentration, such as molarity, and can vary depending on the density and temperature of the solution.

Equivalent weight

Equivalent weight, also known as gram equivalent weight, is a measure of the reactive capacity of a compound in a chemical reaction. It is the amount of a substance that can donate or accept one mole of an electron, a proton, or any other chemical species. Equivalent weight is expressed in grams per equivalent (g/eq) and is calculated by dividing the molar mass of a substance by its valence. The valence of a substance is the number of electrons it can donate or accept in a chemical reaction. For example, the valence of an element is typically the number of electrons in its outermost shell. For compounds, the valence is the charge of the compound divided by the number of atoms that contribute to the charge. For example, the equivalent weight of sulfuric acid (H_2SO_4) is the molecular weight of the acid (98 g/mol) divided by its valence of 2 (since each molecule of H_2SO_4 can donate two protons in a chemical reaction), giving an equivalent weight of 49 g/eq. Equivalent weight is commonly used in acid-base titrations to calculate the amount of acid or base needed to react with a given amount of a substance.

Molarity, Molality and Normality

Molarity, molality and normality are all measures of concentration used in chemistry. Here are their definitions, along with some examples:

Molarity (M): Molarity is the number of moles of a solute dissolved in a liter of solution. The formula for molarity is:

Molarity = moles of solute / liters of solution

For example, a 0.1 M solution of hydrochloric acid (HCl) contains 0.1 moles of HCl per liter of solution.

Molality (m): A molality is the number of moles of solute dissolved in one kilogram of solvent. The formula for molality is:

Molality = moles of solute / kilograms of solvent

For example, a 0.1 m solution of sodium chloride (NaCl) contains 0.1 moles of NaCl per kilogram of solvent (usually water).

Normality is a measure of the concentration of a solution that takes into account the number of acidic or basic protons in a solute. It is defined as the number of equivalents of a solute per liter

of solution. An equivalent is the amount of a substance that can donate or accept one mole of hydrogen ions (H+) in a chemical reaction. The formula for normality depends on the specific solute being used.

Normal solution preparation from salt

$$= \frac{\text{Gram equivalant of substance}}{\text{Volume of solution in L}}$$

To prepare1000mL 1Normal NaOH solution

Molecular weight of NaOH=40

Equivalent weight of NaOH $= \frac{\text{Molecular weight}}{\text{acidity}} = \frac{40}{1} = 40$

When 40 g of NaOH is dissolved in water and final volume of solution is made up to 1L, this solution is known as 1N NaOH solution

Normality with purity correction

Some chemicals are not available commercially as 100% pure, so to get a correct result purity of the chemical has to be considered.

An Example to find Normality of a solution:

If bottle of HCl having purity 35% and Specific gravity 1.19g/mol and molecular weight 35.46. how to prepare 1N of HCl from it?

At first find out the Normality of HCl in the bottle

Normality = Specific gravity x purity% x 1000 / Equivalent Weight

Here, Specific gravity of HCL =1.19 g/ml

Equivalent Weight of HC l=35.46

Purity =35%

$$\text{Normality} = \frac{1.19 \times 35 \times 1000}{100 \times 35.46} = 11.74\text{N}$$

So, the HCl bottle solution has 11.74N

We need to prepare 1N solution in 500ml

Use Formula N1V1=N2V2

where V1 is the volume of the available concentrated acid solution, N1 is its Normality, V2 is the final volume of the diluted solution, and N2 is the desired normality of the diluted solution.

11.74 x V1 = 1 N x 500ml

V1 =1 x 500/11.74=43.59ml

Therefore take 43.59 ml from HCl bottle and make up to 500ml in a standard flask.

We will get 1 N solution.

For Making 1ltr of 0.1N HCl solution

N1 X V1=N2 X V2

12 x V1 =0.1 x 1000ml

V1 =100/12 = 8.3 ml

Therefore take 8.3 ml HCl and make up to 1000ml in a standard flask=0.1N HCl solution

It's important to note that molarity and molality are based on the amount of solute and solvent, respectively, whereas normality considers the reactive capacity of the solute. Each measure has its own specific use, depending on the chemical reaction being studied or the experimental conditions. Relationship between Normality and Molarity From definition of normality.

N = M x n where N refers to normality, M is molarity, and n denotes the number of equivalents.

Table1: Bottle normality and volume requirement to make 1N solution of some common chemicals.

No	Reagent	Approximate			Volume requirement to make 1N solution
		% by weight	Specific gravity	Normality (N)	
1	Hydrochloric Acid	37	1.19	12 N	83 ml
2	Nitric Acid	70	1.42	16 N	63 ml
3	Sulphuric Acid	96	1.84	36 N	28 ml
4	Perchloric acid	70	1.66	11.6 N	86 ml
5	Hydro fluoric acid	46	1.15	26.5 N	38 ml
6	Phosphoric acid	85	1.69	41.1 N	23 ml
7	Acetic acid	99.5	1.05	17.4 N	58 ml

8	Aqueous ammonia	27	0.90	14.3 N	71 ml

PREPARATION OF STANDARD SOLUTIONS

To prepare standard sodium solution from sodium chloride salt.

Take 0.634g accurately weighed dry Analytic quality NaCl. Transfer salt in to 500ml volumetric flask, Dissolve salt using distilled water and make up to the mark.

Atomic weight of Sodium= 23

Molecular weight of NaCl= 58.46

There for 0.634g NaCl contains =0.634 x 23/58.46 =0.25g/Na

Thus, in 500ml of solution We have 250mg Na or 50mg Na/100ml

Ie.500mg/1000ml= 500ppm

ppm (Parts per million) =mg/L

ppb (Parts per Billion) = µg/L

1ppm= 1000ppb

For making 10 ppm working standard from 500ppm stock standard

Formula: N1V1=N2V2

500ppm x v1 =10ppm x 25 (made up volume)

V1=250/500=0.50 ml

Take 0.50 ml and make up to 25 ml in a standard flask to get 10ppm.

ACIDS AND BASES

Acids and bases are two types of chemical compounds that have important roles in many chemical reactions and natural processes.

Acids are substances that release hydrogen ions (H^+) when they dissolve in water, which makes the solution acidic. The more hydrogen ions that are released, the more acidic the solution becomes. Acids have a pH less than 7, with the strongest acids having a pH close to 0. Examples of common acids include hydrochloric acid (HCl), sulfuric acid (H_2SO_4), and acetic acid (CH_3COOH). Acids can have a sour taste and can react with metals, carbonates, and other bases.

Bases are substances that release hydroxide ions (OH-) when they dissolve in water, which makes the solution basic. The more hydroxide ions that are released, the more basic the solution becomes. Bases have a pH greater than 7, with the strongest bases having a pH close to 14.

Examples of common bases include sodium hydroxide (NaOH), ammonia (NH_3), and calcium hydroxide ($Ca(OH)_2$). Bases can have a bitter taste and can react with acids to form salts and water.

Acids and bases can also be defined in terms of their chemical properties. Acids are proton donors, meaning that they donate hydrogen ions to other substances. Bases are proton acceptors, meaning that they accept hydrogen ions from other substances. The strength of an acid or base depends on the extent to which it donates or accepts protons in a chemical reaction.

The concept of acids and bases is central to many areas of chemistry, including acid-base titrations, pH measurements, and buffer solutions. Understanding the properties of acids and bases is important in many fields, including medicine, environmental science, and industrial chemistry.

Dilution of acids

Acids can be diluted by adding water to the concentrated acid solution. Dilution of acids is a common laboratory practice, as concentrated acids can be dangerous to handle and can cause chemical burns if not properly diluted. The process of dilution involves adding a specific volume of water to the concentrated acid solution to reduce its concentration to a desired level. Here is an example of how to dilute a concentrated sulfuric acid solution (H_2SO_4) to a desired concentration:

1. Determine the desired concentration of the diluted acid solution. For example, suppose you want to prepare a 1 M solution of sulfuric acid.
2. Calculate the volume of the concentrated acid solution required to prepare the diluted solution. To do this, use the formula: V1 x C1 = V2 x C2

   where V1 is the volume of the concentrated acid solution, C1 is its concentration, V2 is the final volume of the diluted solution, and C2 is the desired concentration of the diluted solution.

For example, if you have a 12 M sulfuric acid solution and want to prepare a 1 M solution, you would need to use the following equation:

V1 x 12 M = V2 x 1 M

Solving for V1, you get:

V1 = (V2 x 1 M) / 12 M

3. Measure out the calculated volume of the concentrated acid solution using a graduated cylinder or pipette.
4. Transfer the measured volume of the concentrated acid solution to a clean, dry flask.

5. Add the required amount of water to the flask to make up the final volume of the diluted solution. For example, to prepare a 1liter solution, you would add enough water to bring the total volume up to 1 liter.
6. Mix the solution thoroughly by swirling the flask or using a magnetic stirrer.

It is important to use caution when diluting acids, as they can cause chemical burns if they come into contact with skin or eyes. Always wear appropriate personal protective equipment, such as gloves and goggles, and work in a well-ventilated area to prevent the buildup of acid fumes.

TITRIMETRY

Titrimetry is a type of analytical chemistry technique that involves the determination of the concentration of a solution by measuring the amount of a reagent required to react with it. The reagent is typically added to the solution in a controlled manner until the reaction is complete, which can be detected by a change in color or another measurable property. The amount of reagent added is then used to calculate the concentration of the solution. There are several methods of titrimetry, including acid-base titration, redox titration, complexometric titration, and precipitation titration. Here are some examples of each method:

1. Acid-base titration: This method involves the reaction of an acid with a base, or vice versa, in order to determine the concentration of one of the solutions. A common example of acid-base titration is the determination of the concentration of hydrochloric acid (HCl) in a solution. A standardized solution of sodium hydroxide (NaOH) is added to the HCl solution until the reaction is complete, as indicated by a color change in the solution. The volume of NaOH required to neutralize the HCl can then be used to calculate the concentration of the acid solution.
2. Redox titration: This method involves the reaction of a reducing agent with an oxidizing agent, or vice versa, in order to determine the concentration of one of the solutions. A common example of redox titration is the determination of the concentration of iron (II) ions (Fe^{2+}) in a solution. A standardized solution of potassium permanganate ($KMnO_4$) is added to the Fe^{2+} solution until the reaction is complete, as indicated by a color change in the solution. The volume of $KMnO_4$ required to react with the Fe^{2+} can then be used to calculate the concentration of the iron ions.
3. Complexometric titration: This method involves the reaction of a metal ion with a complexing agent in order to determine the concentration of the metal ion. A common example of complexometric titration is the determination of the concentration of

calcium ions (Ca^{2+}) in a solution. A standardized solution of ethylenediaminetetraacetic acid (EDTA) is added to the Ca2+ solution until the reaction is complete, as indicated by a color change in the solution. The volume of EDTA required to chelate all the calcium ions can then be used to calculate the concentration of the metal ion.

4. Precipitation titration: This method involves the reaction of a solution with a reagent that causes a precipitate to form, which can be used to determine the concentration of one of the solutions. A common example of precipitation titration is the determination of the concentration of chloride ions (Cl^-) in a solution. A standardized solution of silver nitrate ($AgNO_3$) is added to the Cl- solution until all the chloride ions have reacted with the silver ions to form a white precipitate of silver chloride (AgCl). The volume of $AgNO_3$ required to react with all the chloride ions can then be used to calculate the concentration of the Cl- solution.

STANDARDISATION OF H_2SO_4

A strong acid is usually standardized with a weak base:

Na2Co3 solution (in flask) is titrated against 0.1 N H_2SO_4. (Burette)

Procedure

0.530 g of sodium carbonate (Na_2CO_3) is made up to 100 ml (0.1N). (Prepare according to the Normality Calculation)

1.5 ml of H_2SO_4 is diluted to 500 ml (0.1N). (Prepare according to the Normality Calculation)

Indicator…Methyl orange

(End point -Light yellow to light orange)

During Titration better to take 3 concordant values

Normality of Na_2CO_3 = (N1)

Volume of Na_2CO_3 = (V1)

Volume of H_2SO_4 used =V2 (titre value)

N 1 V 1 = N 2V 2

$$N2 = \frac{N1V1}{V2}$$

STANDARDISATION OF NaOH

A strong base is usually standardized with a weak acid.

- 4g of NaoH is dissolved in 1000ml of distilled water (0.1 N). (Prepare according to the Normality Calculation)
- 0.63 g Oxalic acid is dissolved in 100 ml (0.1 N). (Prepare according to the Normality Calculation)
- Oxalic acid is taken in burette.
- 10ml NaoH is taken in the flask.
- One drop of phenolphthalein is added and titrated against Oxalic acid.

(End point of titration …Pink to colourless)

During Titration better to take 3 concordant values

Volume of Oxalic Acid used = V1

Normality of Oxalic acid =0.1 N

Volume of NaoH used =V2

Normality of NaOH=N2

$$N\,1\,V\,1 = N\,2V\,2$$

$$N2 = \frac{N1V1}{V2}$$

COMMON UNITS OF CHEMISTRY

It's important to use the correct units when measuring quantities in a laboratory to ensure accuracy and consistency in experimental results. Chemistry laboratories commonly use a variety of units for measurements, including:

1. Mass: The unit of mass in the SI system is the kilogram (kg), but in the laboratory, smaller masses are typically measured in grams (g) or milligrams (mg). Smaller units such as micrograms (μg) and nanograms (ng) may also be used.
2. Volume: The SI unit for volume is the cubic meter (m^3), but laboratory volumes are usually measured in liters (L) or milliliters (mL). Smaller volumes can be measured in microliters (μL) or nanoliters (nL).
3. Length: The SI unit for length is the meter (m), but in the laboratory, smaller lengths are commonly measured in centimeters (cm) or millimeters (mm). Micrometers (μm) and nanometers (nm) are used for even smaller lengths.

4. Temperature: Temperature can be measured in various units, with the most common being degrees Celsius (°C) and degrees Fahrenheit (°F). In the SI system, temperature is measured in kelvin (K).
5. Time: Time is usually measured in seconds (s), minutes (min), and hours (h).
6. Pressure: Pressure is often measured in pascals (Pa) or atmospheres (atm). Other units such as millimeters of mercury (mmHg) and pounds per square inch (psi) may also be used.

BASIC LABORATORY EQUIPMENT AND USAGES

There are several pieces of equipment commonly used in chemistry laboratories, each with a specific purpose. Some of the most common equipment and their uses include:

1. Bunsen burner: A gas burner used for heating, sterilizing, and combustion.

2. Erlenmeyer flask: A conical flask with a narrow neck used for containing and mixing liquids.

3. Test tube: A cylindrical tube used for holding and heating small amounts of liquid or for observing chemical reactions.

4. Beaker: A cylindrical container with a flat bottom and a lip used for mixing and heating liquids.

5. Pipette: A calibrated glass or plastic tube used for measuring small volumes of liquid.

   A volumetric pipette is designed to deliver a single, specific volume of liquid, while a graduated pipette can deliver various volumes of liquid by adjusting the level to which it is filled.

   A micropipette is a more modern and precise type of pipette that can measure and deliver extremely small volumes of liquid, typically ranging from 0.1 microliters to 1000 microliters. Large volume pipettes are also available now. Micro pipettors use disposable plastic tips.

6. Burette: A long, graduated tube with a stopcock at the bottom used for measuring precise volumes of liquid during titrations.

7. Glass rod: A long, thin glass rod used for stirring liquids or solutions.

8. Wash bottle: A plastic bottle with a nozzle used for dispensing small amounts of water or other solvents.

9. Spatula: A flat, thin tool used for transferring small amounts of solid chemicals from one container to another.

10. Desiccator: A container used for drying or storing moisture-sensitive chemicals. It usually has a vacuum seal and can contain a drying agent such as silica gel.

11. Condenser: A glass tube used for cooling hot vapors and condensing them back into a liquid. It is commonly used in distillation or reflux setups.

12. Funnel: A conical or cylindrical device used for transferring liquids or solids from one container to another.

13. Graduated cylinder (measuring vessel): A tall, narrow container used for measuring volumes of liquid.

14. Thermometer: An instrument used for measuring temperature.

15. Balance: An instrument used for measuring the mass of an object or substance.

16. pH meter: An electronic instrument used for measuring the acidity or basicity of a solution.

17. Spectrophotometer: An instrument used for measuring the amount of light absorbed or transmitted by a substance at different wavelengths.

IMPORTANT POINTS TO CONSIDER WHEN WORKING IN A LABORATORY

Here are some tips for conducting successful chemistry lab experiments:

1. Plan ahead: Before beginning any experiment, read the procedure thoroughly and make sure you understand all the steps involved. Prepare all materials and equipment in advance, and make sure you have all the necessary safety gear.
2. Follow instructions carefully: Always follow the experiment procedure exactly as it is written. Do not skip steps, modify procedures, or take shortcuts, as this can affect the accuracy of your results.
3. Use proper safety equipment: Always wear proper safety gear, including gloves, goggles, and lab coats, to protect yourself from hazardous chemicals and materials.
4. Keep a detailed record: Keep a detailed record of all the steps you take during the experiment, as well as any measurements or observations you make. This will help you to analyze your results and identify any sources of error.
5. Be organized: Keep your work area clean and organized, and make sure you have all the necessary equipment and materials readily available. This will help you to work more efficiently and avoid mistakes.
6. Work carefully and accurately: Take your time when performing measurements and handling chemicals, and work carefully to minimize the risk of spills or accidents.
7. Verify your results: Once you have completed the experiment, double-check your results to ensure that they are accurate and consistent. If possible, compare your results with those obtained by other researchers to confirm their validity.
8. Dispose of chemicals properly: Make sure to dispose of all chemicals and materials properly according to the guidelines set by your lab or institution. This will help to minimize the risk of contamination or injury.

Sensory Evaluation of Seafood

Sensory evaluation is done separately for raw and cooked fish. Assessment is based on appearance, color, odor, flavor, taste, and texture (like extremely = 9 to dislike extremely = 1). Samples with no off-flavor, bright color, firm and elastic structure will be given a score of 9–7. Those samples with trace off-flavor, pale to colorless, and elastic structure will be scored from 7 to 5. Samples with medium off-flavor, slight brownish color, and slight soft texture will give a score of 4–5.

For the evaluation of cooked sample, the samples are cooked for 10 min in boiling brine (1.5% NaCl). Then scoring has to be given as per the characteristics. An overall acceptability score is calculated based on the total score obtained for raw and cooked samples. An overall score below four is taken as "rejected."

SENSORY EVALUATION SCORE CARD

(Please score the sample characteristics by placing the relevant score)

Sample: …….…….

Attributes	**Sample I**	**Sample II**
Appearance		
Colour		
Odour		
Texture		
Overall acceptability		

Sample: ……Cooked sample…….

Attributes	**Sample I**	**Sample II**
Appearance		
Colour		
Odour		
Texture		
Overall acceptability		

Please score the sample characteristics according to the following scale

Quality Grade Description	**Score**
Like extremely	09
Like very much	08
Like moderately	07
Like slightly	06
Neither likes nor dislikes	05
Dislike slightly	04
Dislike moderately	03
Dislike very much	02
Dislike extremely	01

Measurement of pH of Fish Muscle (AOAC, 1990)

pH is the concentration of hydrogen (H+) ions in the sample. It is the measurement of acidity or alkalinity of the sample. pH is one of the important biochemical quality indices of fish

The pH of fish muscle was determined by using a digital pH meter. Calibration of pH meter was done by using 4.01, 7.00 and 10.00 buffer solutions respectively at ambient temperature and pressure.

Post mortem pH of fish can vary from 6.0 to 7.1 depending on season, species, and other factors. Generally it is noticed that, the pH is about 6.0–6.5 for fresh fish, and it increases during storage. The limit of acceptability is usually 6.8–7.0.

Sample Preparation:

Weigh 4 g. of homogenized muscle in a beaker and add 40 ml of deionised water and blend. This solution can be used for the measurement of pH using digital pH meter.

Determination of Total Volatile Base Nitrogen (TVBN)

Principle

The volatile compounds produced in fish muscle during post - mortem, are TMA and ammonia. They will escape from the alkaline solution to form a vapor and will dissolve in acid solutions, neutralizing some of the acid. The acid is then titrated with alkali to determine the amount, which has not been neutralized. From this, the volume of acid neutralized by the volatile base is calculated**.**

Reagents and apparatus

10% Trichloroacetic acid (w/v)

0.01N sulphuric acid

0.01N sodium hydroxide

Saturated potassium carbonate

Tashiro's indicator

Procedure

Take 10g sample and homogenize in 100ml 10% TCA solution. Filter using a Whatman No.1 filter paper to remove the filtrate. Make up the extract up to 100ml using10% TCA solution. Pipette 1ml 0.01N standard acid, pour into the inner chamber of Conway unit. Then add 1ml extract and 1ml saturated potassium carbonate into the outer chamber. Immediately seal the unit with a glass lid. Gently rotate the unit to mix the solution in chamber. Keep the unit undisturbed for overnight at room temperature. Then titrate the acid in the central chamber against 0.01N NaOH using 2 drops of Tashiro's indicator. At the end point green color will appear. Prepare a blank in the same way without sample.

Calculation

$$\text{TVBN (mg \%)} = \frac{(A-B) \times 0.14 \times \text{volume of extract} \times 100}{\text{volume of sample taken} \times \text{sample weight}}$$

where,

A = Volume of 0.01N NaOH used up for titration of the blank

B = Volume of 0.01N NaOH used for titration of the sample

DETERMINATION OF TMA

Principle

The volatile compounds produced in fish muscle during post – mortem, are TMA and ammonia. They will escape from the alkaline solution to form a vapor and will dissolve in acid solutions, neutralizing some of the acid. The acid is then titrated with alkali to determine the amount, which has not been neutralized. From this, the volume of acid neutralized by the volatile base is calculated**.**

Reagents and apparatus

10% Trichloroacetic acid (w/v)

0.01N sulphuric acid

0.01N sodium hydroxide

Saturated potassium carbonate

37% formaldehyde

Tashiro's indicator

Procedure

Take 10g sample and homogenize in 100ml 10% TCA solution. Filter using a Whatman No.1 filter paper to remove the filtrate. Make up the extract up to 100ml using10% TCA solution. Pipette 1ml 0.01N standard acid, pour into the inner chamber of Conway unit. Then add 1ml 37% formaldehyde, 1ml extract and 1ml saturated potassium carbonate into the outer chamber. Immediately seal the unit with a glass lid. Gently rotate the unit to mix the solution in chamber. Keep the unit undisturbed for overnight at room temperature. Then titrate the acid in the central chamber against 0.01N NaOH using 2 drops of Tashiro's indicator. At the end point green color will appear. Prepare a blank in the same way without sample.

Calculation

$$\text{TMA (mg \%)} = \frac{(A-B) \times 0.14 \times \text{volume of extract} \times 100}{\text{volume of sample taken} \times \text{sample weight}}$$

where, A = Volume of 0.01N NaOH used up for titration of the blank

B = Volume of 0.01N NaOH used for titration of the sample

Determination of Thio Barbituric Acid number (TBA)

The TBA value was measured by the method described by Tariadgis *et al*. (1960).

Reagents

1. 4 N HCl
2. TBA reagent: 0.2883 gm TBA reagent was dissolved in 90% acetic acid by slight warming and the volume was made upto 100 ml with 90% acetic acid.

Procedure

10 gm of the fish sample was blended with 50 ml of distilled water using a mortar and pestle. The mixture was washed out into a 250 ml flat bottom flask with 47.5 ml distilled water. 2.5 ml of 4 N HCl was then added to the flask. The flask was then connected to a distillation unit and was heated by an electric mantle in such a way that 50 ml of the distillate was collected in 10 minutes. 5 ml of the distillate was taken in a stopperd tube and 5 ml of the reagent was added. The tubes wee then placed in boiling water for exactly 35 minutes. Blank was prepared by using 5 ml distilled water and 5 ml TBA reagent. After cooling the optical density was measured at 538 nm by a UV spectrophotometer. TBA number, expressed as mg malonaldehyde / Kg sample = 7.8 × optical density.

Microbial quality indices of seafood

For routine sampling of fish/prawn, the following microbiological parameters are determined.

Parameter	Medium
i) Total plate count (TPC)	PCA
ii) Total Enterobacteriaceae count (includes all coliforms and *Salmonella* & *Shigella*)	VRBGA
iii) *Escherichia coli (E. coli)*	T7
iv) *Staphylococcus aureus*	BP
v) Faecal streptococci	KF

Method:

25 g of the sample is aseptically cut into a sample dish and macerated with 225 ml phosphate buffer (PB) in a sterile mortar and blended in a stomacher blender.

Sampling scheme

25 g sample + 225ml PB 10^{-1} dilution

1ml + 9ml PB 10^{-2} dilution (0.5 ml to BP, T7;1 ml to KF, VRBGA)

1ml + 9ml PB 10^{-3} dilution
(0.5ml to BP, T7 ;1ml to KF, VRBGA and PCA)

1ml + 9ml PB 10^{-4} dilution (1ml to PCA)

1ml + 9ml PB 10^{-5} dilution (1ml to PCA)

1) For PCA

Pour plating on PCA. 1 ml of 10^{-3}, 10^{-4} and 10^{-5} dilutions in duplicate.

2) For KF and VRBGA, pour plating technique is followed. One ml each of 10^{-2} and 10^{-3} dilutions are plated (Note: After cooling the molten KF to 45°C, add 1 ml of 0.1% TTC per 100 ml medium).

3) For T7 and BP, plates have to be prepared as follows:-

a) One 100ml flask of Tergitol-7 agar (T7) is melted in a water bath, cooled to about 50°C and aseptically added 0.25ml of 1% sterile TTC solution. Pour into sterile petridishes (15-20 ml each), allow to set and dry at 56°C for 45min. Cool to room temperature (RT).

b) One 100ml flask of Baird-Parker medium (BP) is melted & cooled to about 50°C; aseptically add 1ml of sterile 1% potassium tellurite solution, followed by 5ml of 50% egg yolk emulsion. Mix well, pour into sterile petridishes, allow to set; dry at 56°C for 45mincool to RT.

Arrange 6 petridishes (in duplicate) for PCA & 4 each for KF and VRBGA (in duplicate). Also, arrange 4 plates each of pre-set T7 and BP agar in duplicate. Label appropriately, viz: sample name, dilution, medium and date.

For PCA, KF and VRBGA plates, 1ml each of the appropriate dilutions are pipetted and pour plated with the corresponding medium. Plates are allowed to set, inverted and incubated at 37°C.

For T7 and BP plates, 0.5ml each of the appropriate dilutions are surface plated using sterile bent glass rod. Plates are inverted and incubated at 37°C for 18-24 h. The plates are examined after the incubation.

Observe VRBGA and T7 plates after 18-24h.

On VRBGA- Red, small (2-4 mm dia) colonies are counted as Enterobacteriaceae colonies. Take average count of duplicate plates.

Total Enterobacteriaceae count / g = Average count x dilution factor.

On T7 plates: *E. coli* colonies are lime yellow, occasionally with rust brown centre and an yellow zone around (Note:Yellow slimy raised or convex colonies are not to be considered as *E. coli* colonies). Take average of duplicate plates.

E. coli/g = Average count x 2 x dilution factor.

Average count is multiplied by two because only 0.5ml of the sample dilution was added to the plates.

Note: *E.coli* does not reduce TTC. So colonies with a red tinged center should not be confused for *E.coli.*

Confirmation of *E. coli*

The lime yellow colonies, occasionally with rust brown centre and an yellow zone around, on T7 plates are counted as *E. coli*. However, to confirm them as *E. coli*, the following procedure has to be adopted.

1. Streak on EMB agar

EMB agar (Eosine-Methylene blue agar) is melted, cooled to 50°C, poured into petri dishes and allowed to set. The set plates are dried at 56°C for 45 min and cooled to room temperature. Typical yellow colonies from T7 plates are picked with a sterile platinum loop and streaked on to EMB plates, by the streak-dilution method. Incubated at 37°C for 18-24 h. Well isolated colonies of 2-3mm dia with a greenish metallic sheen by reflected light and dark purple centre by transmitted light is picked and subcultured on TGA slants and incubated at 37°C for 18-24 h.

2. IMVIC tests

From the TGA slants above, inoculate to the following media.

a) Tryptone broth (Indole medium)

Inoculate a little of the culture to Tryptone broth and incubate at 37°C for 48 h.

b) MRVP medium

Inoculate each culture into 2 tubes of MRVP medium and incubate at 37°C for 48 h.

c) Simmon's Citrate agar

Streak a little of the culture to Simmmon's citrate agar slants and incubate at 37°C for 48 h.

3 Results:

Observe results after 48 h of incubation (Refer Media Section for tests)

1. Tryptone broth

Test for indole production using Kovac'sindole reagent. A red or pink colour at top indicates +ve test.

2. MRVP medium

a) Into one tube, add Methyl Red indicator. A redcolour indicates +ve MR test.

b) Using 1 ml of the culture from the second tube, do the VP test as described in the Media section. Eosine pink colour indicates +ve VP test.

3. Simmon's Citrate agar

Growth indicated by a change in the colour of the medium in the innoculated tubes from green to blue indicates a +ve test for citrate utilisation by the bacterial culture.

A culture giving the following results is confirmed as *E. coli*

Indole +ve

Methyl Red +ve

VP -ve

Citrate -ve

Note: (Eijkman's test): This is used as an optional confirmatory test for *E.coli*.

E. coli cultures will grow and produce gas in EC broth at 44.5±0.5°C in 24-48h.

On BP agar plates: Observe after 36-48 h. *Staphylococcus aureus* colonies are black with thin white margin and a zone of clearance around.

Total *S. aureus* count / g = Average count x 2 x dilution factor.

Average count is multiplied by two since the quantity of sample dilution added is only 0.5 ml

Confirmation of Staphylococcus aureus.

S. aureus is confirmed by Coagulase test. DifcoBacto-coagulase -EDTA is used for the test. To 0.5 ml of coagulase reagent in a small sterile test tube, add 2 drops of 24 h old bacterial culture (grown in Brain heart infusion broth); incubate in a serological water bath at 37°C. Observe every 30 min upto 4 h Coagulation (jell formation) of the contents of the tube indicates a +ve reaction for coagulase.

Alternatively, Mannitol Salt Agar (MSA) can be used for confirmation, where Coagulase test reagent is not available. Divide the mannitol salt agar plates into 2 or 4 areas. Inoculate on the surface of the marked area, a heavy inoculum of the suspected Staphylococcus culture. Incubate at 36+/- 1C for 36 to 48 h. Examine the colonies. Yellow colonies surrounded by bright opaque yellow zones indicate Coagulase +ves. Non-pathogenic *Staphylococcus* gives colonies with red or reddish purple or purple zone (Negative reaction).

On KF Agar plates: Observe after 36-48 h. Count all surface and sub-surface red to pink colonies (some will be with a thin white margin) as faecal streptococci.

Confirmation of faecal streptococci.

Faecal streptococci (Enterococci) group includes Streptococcus faecalis and *Streptococcus faecium. They are confirmed by catalase test.*

Pick 5-6 typical colonies from KF agar to BHI broth and incubate at 36 ±1°C for 24-48h. Mix 3ml of the culture with 0.5 ml of dilute Hydrogen peroxide (H_2O_2). (Usually H_2O_2 is available as 30 vol / vol solution (30%). Dilute 2ml to 5ml for the test). Note for evolution of gas bubbles (oxygen). No evolution of gas bubbles indicates a negative reaction. Faecal streptococci are catalase negative. So a negative result in catalase test confirms faecal streptococci.

Bacterial DNA Isolation and Polymerase Chain Reaction

DNA ISOLATION

Heat breaks open bacterial cells and releases cell components such as DNA. Once DNA is released from the cells, PCR can be used to amplify any antibiotic resistance genes present in the sample DNA.

Procedure

1. Label the top and side of your clear PCR tube with your sample number.
2. Use the micropipette to add 1ml of sterile DNAase-free water to the tube.
3. Use a sterile pipette tip to 'touch' a series of bacterial colonies on your plate or overnight grown culture from slants. You can sample 10 colonies, but avoid picking up a large amount a bacterial cells. Too much cellular material will inhibit the PCR reaction.
4. Add 0.5 ml of Tris-EDTA buffer to the bacterial culture
5. Close the top of the tube and flick the bottom to mix your bacterial cells.
6. Heat the tube: Place the tube in the heating plate and heat the cells to 95°C for 5 minutes.
7. Immediately transfer the tubes to -20^{0}C.

POLYMERASE CHAIN REACTION

Polymerase chain reaction, or PCR, is an *in-vitro* technique used to amplify particular segments of DNA found in a sample. PCR is used to rapidly produce billions of copies of one or more "target sequences" such as genes or repeating sequences of DNA. Once amplified, the target sequences can be used in a variety of ways. For example, DNA segments copied by PCR can be used for gene detection, gene sequencing, or even be inserted into the DNA of another organism. First, the DNA isolated from a particular sample is placed in a test tube with a "mastermix" containing 1) **Taq polymerase** (a heat-stable DNA polymerase), 2) **primers** specific to the target sequence, 3) **a supply of A, C, T, and G nucleotides**, and 4) **buffers** and **salts** necessary for Taq polymerase to function.

Then, the test tube is placed in a PCR machine, which performs the following steps.

1. **Denaturation**: DNA sample is heated, which breaks the hydrogen bonds and separates the DNA strands.
2. **Annealing**: DNA sample is cooled, which allows primers to hydrogen bond (anneal) with complementary sequences in DNA sample.
3. **Extension**: Once primers anneal to DNA sample, the sample is warmed again so Taq polymerase may add nucleotides to the 3' end of the primer according to base-pairing rules.

The PCR machine repeats these 3 steps for 20 or more cycles to yield over a billion copies of the target sequence ***if it is present*** in your DNA sample.

Procedure

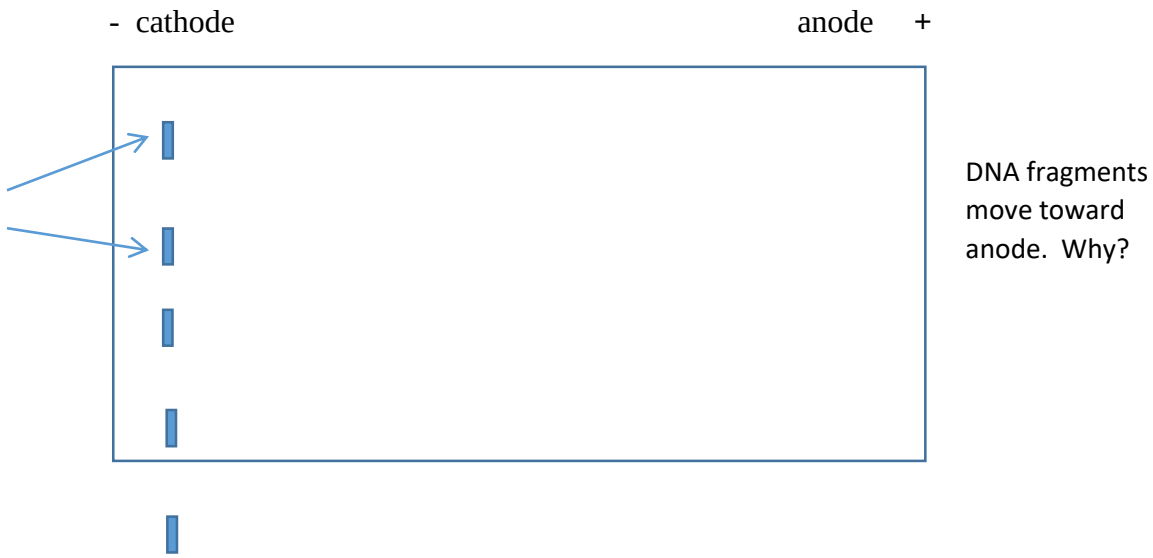
1. Obtain one PCR tube containing the 22 µl PCR mastermix with primers that target the antibiotic reistant gene. Also obtain one PCR tube containing 22 µl PCR mastermix with primers that target the genes.
2. Use the micropipette to transfer 3 µl of template DNA to the mastermix PCR tube.
3. Keep your PCR tubes on ice tray.
4. The PCR tubes will go into the thermocycler, which will control the temperature shifts necessary for the PCR reaction. These shifts will be repeated 20-35 times to yield a large number of copies of the DNA region between the primers (if they are present).
5. After the PCR reactions are completed freeze store your samples for analysis.

GEL ELECTROPHORESIS

PROCEDURE

Gel electrophoresis is a technique that is used to separate fragments of DNA based on their sizes. DNA samples are loaded onto one end of an agarose gel that is immersed in buffer, and an electric current is used to pull the DNA through the gel. Because DNA is negatively charged, the DNA fragments will move away from the negatively-charged cathode and toward the positively-charged anode. Because the DNA fragments must pass through small pores within the agarose gel, the smaller fragments of DNA are able to move through the gel more easily and quickly. After a sufficient period of time has passed, the electric current will have separated the DNA fragments so that the larger fragments remain closer to the start point, while the smaller fragments have migrated farthest in the gel.

DNA



PROCEDURE:

I. PRACTICE LOADING THE GEL (at your benches)

a. Load 20 µl of methylene blue dye by using a micropipette. Wipe the outside of the pipette tip with a paper towel to remove excess dye.
b. Use one hand to brace the wrist of your hand holding the micropipette.
c. Carefully position the tip of the micropipette just at the top of one of the gel's wells.
d. While holding the tip steady over the well, depress the plunger slowly and steadily to push the sample out. Only depress to the first stop, or you risk blowing your DNA/dye out of the well.
e. The dye in the sample will allow you to see where the sample goes. It should drop down for the pipette tip into the bottom of the well (the samples are denser than the buffer in the gel box, so the samples sink to the bottom).
f. Keep the plunger depressed to the first stop as you slowly remove the micropipette from the solution. If you release the plunger before you remove the micropipette from the well, you risk sucking the DNA/dye back out of the well.

II. LOADING THE GELS WITH YOUR DNA SAMPLES

a. Two percent agarose gels have been placed in gel electrophoresis units and covered with a buffer solution. The gel is now ready to be loaded with PCR DNA samples.
b. Load the 3 µl of DNA size standard (100bp DNA ladder) into the first well of each row of wells.
c. Use the micropipette to load 12 µl of the PCR sample to the assigned well in gel.

III. RUNNING THE GEL

a. Once samples have been loaded, the lid is attached to the gel box, taking care that the Black and Red ends match up.
b. The lead wires are then connected to the power supply, taking care that the Black wire connects to the Black port, and the red wire connects to the red port.
c. With the power on and the voltage set at 80-90 volts, the gel is run until the dye in your samples has reached the end of the gel (about 40 minutes).
d. Turn off the power and disconnect the leads from the gel.

IV. INTERPRETING THE GEL

a. Transfer the gel to a UV light box. Do **NOT** turn it on until you have placed the cover/shield over your gel.
b. Follow the instructor's directions on how to observe the gel.
   CAUTION: **The UV light produced by the box is extremely intense. NEVER look at a transilluminator without a UV shield for your eyes. Also do not expose your bare skin to the light produced by the transilluminator.**
c. Take pictures of the gel to allow for further interpretation.
d. Find the well and determine whether the target antibiotic resistance genes were present in the sample.
   i. First, determine if there are any bands present. If so, you must make sure they are the appropriate size.
   ii. If a band of the expected size in the lane, the sample contained that particular antibiotic resistance gene.
   iii. Record your results (+ or – for each gene) in the spreadsheet at the front of the room.

I. GEL ELECTROPHORESIS INTERPRETATION

Ethidium bromide, a dye that binds to DNA molecules and fluoresces orange when exposed to UV light, is usually added to the agarose gel. Upon completion of gel electrophoresis, the gel is exposed to UV light, and DNA bands of different sizes may be observed. The sizes of these bands are determined through comparison with the DNA ladder that is run along with your DNA samples of interest.