Handbook of Seafood Quality, Safety and Health Applications

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Preface

There has been a growing demand for seafoods due to their perceived health benefits Seafoods are highly nutritious and provide a wide range of health-promoting compounds. Safety and quality are two main issues when considering seafoods, since they are highly perishable products, hence special attention should be paid from the time of the catch to the time they are prepared for food and consumed. Safety and freshness/quality of seafoods can be measured by sensory, non-sensory (chemical/biochemical, physico-chemical, and microbiological/biological), and statistical methods. During the last decade, the situation has changed dramatically in the seafood area and there has been a rapid development in the fiel for all three mentioned techniques, some of which are rapid and non-destructive in nature.

The marine ecosystem is the richest source of life, accounting for more than 80% of living organisms. Therefore, utilisation of marine resources (fish marine mammals, micro- and macroalgae, shellfish and invertebrates) for the development of nutraceuticals and functional foods is a daunting and challenging task. Marine-based nutraceuticals are gaining recognition due to their unique features, which are not found in terrestrial-based bioresources. For example, fish marine mammals, and algae are the richest sources of long-chain omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA), which play an important role for health promotion and disease risk reduction. There are over 8,000 published papers on the health benefit of EPA, DPA, and DHA. The role of omega-3 PUFA, in a wide range of products and in the prevention of cardiovascular disease and in the development and function of the brain, has been well demonstrated.

This book is divided into three sections preceded by an introductory chapter (Chapter 1) providing an overview of seafood quality, safety, and health applications. The firs section (Chapters 2–15) describes different aspects of seafood quality; the second section (Chapters 16–28) covers the safety of seafoods; and the fina section (Chapters 29–43) discusses the health applications of seafood products, particularly marine nutraceuticals and functional foods. Contributing to this volume are internationally renowned researchers who have provided a diverse and global perspective of the issues of concern to seafood quality, safety, and health applications. The book will serve as a resource for those interested in the potential application of new developments in marine nutraceuticals and functional foods, as well as the role of science and technology in ensuring safety and quality. Biochemists, chemists, food scientists/technologists, nutritionists, health professionals, and marine technologists, from academia, government laboratories, and industry will benefi from this publication. Although this book is intended primarily as a reference book, it also summarises the current state of knowledge in key research areas and contains ideas for future work. In addition, it provides easy-to-read text suitable for teaching advanced undergraduate and post-graduate courses.

We are indebted to the participating authors for their state-of-the-art contributions and dedication in providing authoritative views resulting from their latest investigations on different aspects of seafood quality, safety, and health applications.

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1 Seafood quality, safety, and health applications: an overview

Cesarettin Alasalvar, Fereidoon Shahidi, Kazuo Miyashita, and Udaya Wanasundara

1.1 Introduction

In 2007, the world's fis production was around 145 million tonnes, valued at approximately US$92 billion. Of the total amount of production, approximately 75% was used for human consumption and the remaining portion used to produce fis meal and fis oil or discarded [1,2]. With more than 30,000 known species, fis form the largest group in the animal kingdom used to produce animal-based foods. Only about 700 of these species are commercially fishe and used for food production [3]. Moreover, several species of crustaceans, molluscans, and seaweeds, as well as microalgae, are used as food for humans. Devising strategies for full utilization of seafoods and their by-products to produce value-added novel products (e.g. long chain omega-3 (n-3 or ω-3) fatty acids, specialty enzymes, protein hydrolysates, peptides, chitin/chitosan, glucosamine, squalene, collagen, carotenoids, etc.) is of great interest.

Some important aspects such as quality, safety, and health effects of seafoods are considered in this book. These factors contribute to optimal utilization of the marine resources together with the consequent maximization of health benefits This overview chapter highlights these important aspects of seafoods.

1.2 Seafood quality

When seafoods are consumed, their quality is perceived through the conscious or subconscious integration of their sensory or organoleptic characteristics. These characteristics may be grouped as appearance, odour, fl vour, and texture [4]. In most cases, the firs opportunity to evaluate the quality of seafood is governed by its appearance. This is true whether we see the fresh product through a display counter or in a packaged container. Much of the favourable response to the appearance of seafood may be achieved by selecting proper packaging and display.

The odour of freshly caught fis is mild and described as typical of the "sea" and "seaweed". If fis is held in ice from the time of catch, it retains its high quality for about one week or longer. During this period, no objectionable "fis y" odour develops [5]. However, long-term
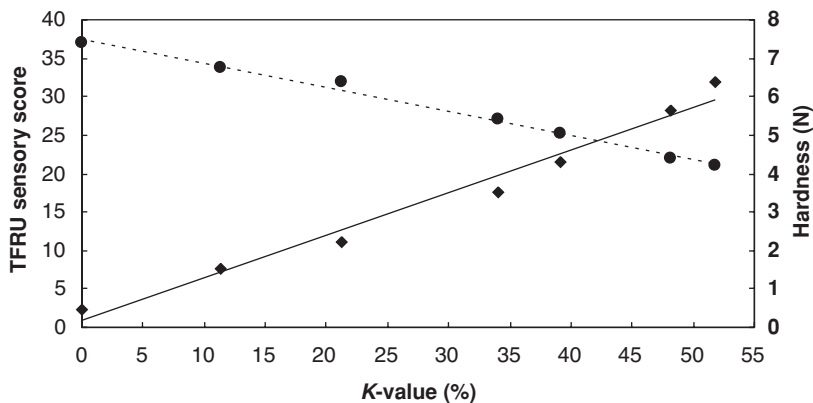
r^2 values of linear regressions are 0.98 (between FTRU and K-value) and 0.99 (between Hardness and K-value)
Maximum demerit points for TFRU sensory score: 38
Unaccaptable limit: TFRU sensory score (20–25), K-value (35–40%), and Hardness (5.0–5.5 N)

Fig. 1.1 Time-independent relationship between the Tasmanian Food Research Unit (TFRU) scheme and K-value and between the hardness and K-value over the storage period. Adapted from Alasalvar *et al.* [12]. With kind permission of Springer Science and Business Media.

storage may lead to the development of an undesirable "fis y" odour due to the formation of trimethylamine (TMA), dimethylamine (DMA), total volatile base nitrogen (TVBN), ammonia, volatile sulphur compounds, and other undesirable compounds characteristic of microbial spoilage [6–11] Several other chemical methods are currently in use for the quality assessment of seafoods [11,12]. Of these, biogenic amines [13,14], adenosine 5′-triphosphate (ATP)-breakdown compounds, and K-related values (Ki, G, Fr, H, and P-values) [15,16] are the most common and provide accurate quality indices. Figure 1.1 shows the correlation between K-value, sensory scores, and hardness [12]. In addition to the above mentioned oxidation products, unsaturated fatty acids present in seafoods can lead to a wide range of lipid oxidation products such as peroxides, carbonyls, aldehydes, alcohols, and ketones, and their interaction compounds that contribute to the odour of the stored seafoods [17]. Table 1.1 shows the various carbonyl compounds derived via lipid oxidation in fis tissues.

Fatty fis such as mackerel, herring, salmon, and sardines have more fl vour than lean fis such as cod, haddock, and hake. The fl vour of fatty fis is pleasant as well as unique, but only while the quality is good. However, due to high fat content, these fis can undergo rapid

Table 1.1 Volatile carbonyl compounds derived via lipid oxidation in fish tissues

Compounds	Origin	Flavour note	Reference
4-Heptenal	n-3 PUFA	Creamy	[58]
2,4-Heptadienal	n-3 PUFA	Rancid hazelnut	[58]
2-Hexenal	n-3 PUFA	Green grass	[59]
2,4,7-Decatrienal	n-3 PUFA	Oxidized fish oil	[60]
1-Octen-3-ol	n-6 PUFA	Mushroom, melon-like	[59]
1,5-Octadien-3-ol	n-3 PUFA	Mushroom, seaweed	[59]
2,5-Octadien-1-ol	n-3 PUFA	Mushroom, seaweed	[59]
1,5-Octadien-3-one	n-3 PUFA	Mushroom	[59]
2-Nonenal	n-6 PUFA	Cucumber-like	[59]
2,6-Nonadienal	n-3 PUFA	Cucumber-like	[59]

Abbreviation: PUFA, polyunsaturated fatty acids.

oxidation and develop rancid/oxidized fl vours that are objectionable to most people. The off-fl vours that develop in the different species have different effects on the organoleptic acceptability of the products [4].

The fina criterion used in the organoleptic evaluation of seafood is texture, which is related to the physical properties that are experienced during biting and chewing. Although this criterion is more relevant when applied to cooked fish texture tests are made routinely by inspectors on raw fish because that is a good indicator of the texture of cooked seafood.

Crude marine oil is a by-product of the fis meal industry and is considered a good source of nutritionally important long-chain n-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, crude oil should be further processed to improve its quality characteristics as well as its shelf-life [18]. The basic processing steps of crude marine oil are degumming, alkali-refining bleaching, and deodorization [19]. During processing, impurities such as free fatty acids (FFA), mono- and diacylglycerols (MAG and DAG), phospholipids, sterols, vitamins, hydrocarbons, pigments, proteins and their degradation products, suspended mucilaginous compounds, and oxidation products of fatty acids are removed from the crude oil. Processing of marine oils is similar to that of vegetable oils; however, the quality of crude marine oils is less uniform than crude vegetable oils. High quality crude oils may be obtained by proper handling of raw material, such as minimizing damage to fis and proper chilling after landing [20]. The degree of unsaturation of the fatty acids makes them extremely vulnerable to oxidative degradation [21,22]. Volatile compounds generated upon oxidation of such fatty acids contribute to the unpleasant fl vours and odours of the oil and the food products containing such oil. Oxidation of the double bonds in unsaturated fatty acids in the oil can occur in the basic processes of autoxidation, photo-oxidation, and thermal oxidation [23]. A basic knowledge of these oxidation processes is required to understand the mechanism of the deterioration of the quality of food grade fis oil. The nature of oxidation, as well as to what extent this occurs, depends upon the chemical structures of the fatty acids involved, and other constituents, even if in minor quantities in the product, as well as the conditions of handling, processing, and storage. Physical factors such as the surface area exposed to oxygen, oxygen pressure in the surrounding environment, temperature, and irradiation can contribute to the oxidation of fatty acids [24]. The origin of the off-fl vours is in the breakdown products of hydroperoxides of the highly unsaturated lipids in fis and/or fis oil.

In this book, several approaches are described to protect unsaturated fatty acids from oxidation. Extreme care must be practised, especially during handling, processing, transferring and transporting, packaging, and storage of oil, to minimize oxidation through exposure to unfavourable conditions. High temperatures should be avoided in processing and the fis or fis oil should never be exposed to oxygen and light. Processed oil containing unsaturated fatty acids should be stored in the dark, at or below $-20°C$, under an inert gas such as nitrogen or argon. Besides preventive measures, antioxidants and related compounds also can be used to retard the oxidation of unsaturated fatty acids in fis oil. These compounds may have different inhibitory activities in the protection of oils against the oxidation process. Microencapsulation of fis oil into a stable fl wable powder extends the shelf-life and prevents the oxidative deterioration of unsaturated fatty acids [25].

1.3   Seafood safety

Quality and safety are important parameters for perishable foods such as fis and fis products. About one-third of the world's food production is lost annually as a result of

microbial spoilage [26]. Food safety cannot be assured by inspection alone and knowledge of factors that influenc growth, survival, and inactivation of pathogenic micro-organisms is an essential element in the design of processing, storage, and distribution systems that provide safe seafoods [27].

The fles of healthy and live fis is generally thought to be sterile, as their immune system prevents the growth of bacteria [28,29]. When the fis dies, the immune system stops functioning and bacteria can proliferate freely. Bacteria can be either of the spoilage type or the pathogenic type. Spoilage is define as the sensory changes resulting in a fis product being unacceptable for human consumption. It is caused by autolytic and chemical changes or off-odours and off-fl vours due to bacterial metabolism [28,30]. Some of the major spoilage bacteria in seafood are *Pseudomonas* spp., H_2S-producing bacteria, *Shewanella* spp., *Enterobacteriaceae*, lactic acid bacteria, *Photobacterium phosphoreum*, and *Brochothrix thermospacta* among others [30–37]. Pathogenic bacteria associated with seafood can be categorized into three general groups:

1) bacteria (indigenous bacteria) that belong to the natural microflor of fis (*Clostridium botulinum*, pathogenic *Vibrio* spp., *Aeromonas hydrophila*);
2) enteric bacteria (non-indigenous bacteria) that are present due to faecal contamination (*Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*); and
3) bacterial contamination during processing, storage, or preparation for consumption (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens, Salmonella* spp.) [30,38–40].

Standard (traditional) methods for recovering micro-organisms from seafood include enrichment culture, streaking out onto selective or differentiating media or direct plating onto these, and identificatio of colonies by morphological, biochemical, or immunological tests [41]. These methods require a lot of human labour, are costly, and usually take between two and fi e days. In contrast to standard methods, molecular methods allow the rapid detection and identificatio of specifi bacterial strains and/or virulence genes without the need for pure cultures. They are mainly based on oligonucleotide probes, polymerase chain reactions (PCR), or antibody techniques [30,41–43]. The use of probes and PCR in seafoods has increased dramatically in recent years. Gene probes and PCR primers for detecting and identifying almost every food-borne pathogenic bacterial species have been developed.

As mentioned above, when harvested in a clean environment and handled hygienically until consumption, fis is very safe. Unfortunately, unhygienic practices, including insufficien refrigeration and sub-standard manufacturing practices, can be at the origin of many outbreaks of fish-born illnesses. Fish-borne illnesses can be broadly divided into fish-born infections and fish-born intoxications (Table 1.2). In the firs case, the causative agent (bacteria, viruses, or parasites) is ingested alive and invades the intestinal mucous membrane or other organs (infection) or produces enterotoxins (toxi-infection). Protection from the environment, personal hygiene, education of fis handlers, and water treatment (e.g. chlorination) are therefore essential in the control of fish-born diseases. In the case of intoxications (microbial, biotoxin, and chemical), the causative agent is a toxic compound that contaminates the fis or is produced by a biological agent in the fish If the agent is biological, intoxication can occur even if the agent is dead, as long as it has previously produced enough toxins to precipitate the illness symptoms [2].

Table 1.2   Types of fish-borne illness. Adapted with permission from FAO [2]

Types of illness		Causative agent
Infections	Bacterial infections	*Listeria monocytogenes, Salmonella* spp., *Escherichia coli, Vibrio vulnificus, Shigella* spp.
	Viral infections	Hepatitis A virus, Norovirus, Hepatitis E.
	Parasitic infections	Nematodes (round worms), Cestodes (tape worms), Trematodes (flukes)
	Toxi-infections	*Vibrio cholerae, Vibrio parahaemolyticus, E. coli, Salmonella* spp.
Intoxications	Microbial	*Staphylococcus aureus, Clostridium botulinum*
	Biotoxins	Ciguatera, Paralytic shellfish poisoning (PSP), Diarrheic (DSP), Amnesic (ASP), Neurotoxic (NSP), Histamine
	Chemical	Heavy metals: Hg, Cd, Pb. Dioxines and polychlorinated biphenyls (PCBs). Additives: nitrites, sulphites

1.4 Health applications of seafood

The unique and phenomenal biodiversity of the marine environment contributes to the presence of a large pool of novel and bioactive molecules. Epidemiological studies have established a positive correlation between marine food consumption and a reduced risk of common chronic diseases such as cardiovascular disease (CVD) and cancers [44–48]. The health beneficia effects of some marine bioactives have been made clear on the basis of nutritional and nutrigenomic studies [49–53]. Thus, dietary marine products are expected to prevent several diseases. Although perception of the term "marine nutraceuticals" to the health care professionals and consumers is still largely limited to popular fis oils rich in highly unsaturated n-3 fatty acids, research has also been shifted to other marine bioactives such as collagen, peptides, chitin, chitosan, chitosan oligomers, glucosamine, carotenoids, and polyphenols, etc. Exciting developments in nutrigenomics and the human genome project, combined with formulation of food products containing specifi marine bioactives, will create new industrial opportunities for food and pharmaceutical companies. Advances in biotechnological processes and their application to the food industry have resulted in commercial success, as seen in the case of glucosamine [54] and collagen [55]. Therefore, we have strong expectations for the further growth of both research and commercialization of marine nutraceuticals and marine functional foods.

In earlier days, fis sources appeared to be inexhaustible and by-products arising from fis processing were considered worthless and routinely discarded. The discovery and development of marine nutraceuticals has changed the commercial value of fisherie processing by-products. Various fis and shellfis source materials such as skin, scales, frame bones, fins visceral mass, head, and shell are now utilized to isolate a number of bioactive commodities. Marine algae, including micro- and macroalgae, are also good resources for other marine bioactive materials (Table 1.3).

Marine lipids generally contain a wider range of fatty acids than terrestrial plants and animals [56]. Omega-3 polyunsaturated fatty acids (PUFA), such as EPA and DHA, are typical of marine lipids, whereas n-6 PUFA, mainly linoleic acid (LA), is predominant in common vegetable oils. The importance of EPA and DHA in human health promotion has been confirme through research. Although many papers have been published on the health beneficia effects of EPA and DHA, there is still an increased level of interest in nutritional

Table 1.3 Main marine functional materials, sources, and health effects

Marine nutraceuticals	Resources	Health effects	References
PUFA EPA DHA	Marine fin fish and their discards Crustacean shellfish and discards Micro-and macroalgae	Antiatherosclerotic Improvement of cardiac health Hypochloesterolemic Anticancerous Improvement of brain functions, ocular health, and bone health Reduces risk of diabetes Improves blood pressure related risks	[52]
Marine protein hydrolysates and peptides	Marine fin fish and their discards Crustacean shellfish and discards Micro-and macroalgae Mollucsan shellfish and discards	Antihypertensive Anticancerous Antioxidative Reduce anxiety related problems Immune system stimulation Improves blood circulation Hypochlesterolemic	[61,62]
Chitin/chitosan/glucosamine	Crustacean shellfish and discards	Antiarthritic (prevents osteoarthritis) Antitumour Antibacterial Biopolymers for drug delivery	[54,63,64]
Collagen/collagen peptides	Marine fin fish and their discards	Protection of skin photo-aging Antihypertension	[55]
Carotenoids Astaxanthin Fucoxanthin	Marine fin fish and their discards Crustacean shellfish and discards Micro-and macroalgae	Prevents cancer related risks (anticancerous) Relieves from oxidative stress (antioxidant) Antiobesity Antidiabetic Hypocholesterolemic Improves ocular health Improves membrane functions	[65]
Chondroitin sulphate	Marine fin fish and their discards	Antiarthritic (prevents osteoarthritis), Antihypertensive	[54]

Abbreviations: PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; and DHA, docosahexaenoic acid.

and health related issues associated with EPA and DHA as well as other highly unsaturated fatty acids, such as stearidonic acid (SA; 18:4 n-3) and docosapentaenoic acid (DPA; 22:5 n-3).

Marine foods and their processing discards/by-products, micro- and macroalgae, and marine microbes are major potential sources of EPA and DHA. They are also important sources of other functional biomaterials such as proteins, enzymes, vitamins, essential minerals, antioxidants, and pigments. Although the edible portion of these marine resources should be used for food, under- and less-utilized fisher resources and processing by-products of fin-shel fis species have tremendous potential for the recovery of marine nutraceuticals. Thus, there is strong incentive to utilize effectively and economically discard materials for

the recovery of value added products such as marine oils. Annual discards from the fis industry is estimated to be at least 25 to 30% of global fis production [57].

Fish processing discards are potential raw materials, not only for fis oil but also for bioactive proteins/peptides and enzymes. Under-utilized micro- and macroalgae are also good resources for bioactive peptides together with functional polysaccharides. Most marine proteins are used as nutraceutical ingredients after hydrolysis, except for collagen, which is the major structural component of skin, bone, tendon, and cartilage of animals and is employed in cosmetics, biomedical, and pharmaceutical industries. Given the proper methods, much higher quality and more functional low-molecular-weight products could be produced from marine proteins. A number of methods have been proposed for this purpose, the most general one being enzymatic hydrolysis to produce bioactive peptides with different molecular weights and properties.

Enzymatic processes are also used for the production of glucosamine from chitin found in shellfis discards. Solid wastes from processing of crustaceans provide an important source for industrial production of chitin. Glucosamine is produced from chitin on the basis of chemical processing, but more attention has been paid to their enzymatic production. Recently, the production of chitin oligomers has been the focus of research. Glucosamine, which is one of the most thoroughly studied marine nutraceuticals with a big market share, is a precursor for glycosaminoglycans that are a major component of joint cartilage.

1.5   Conclusions

Traditional methods for assessing seafood quality have a limited place in current practices of quality assurance of seafood products. The measurement of the *K* and other related values based on ATP breakdown is considered to be one of the best techniques for evaluating freshness of fis stored at temperatures above freezing. These values correlate well with the sensory scores. In addition, rapid analytical techniques using sophisticated instruments, including visible and near infrared (VIS/NIR), electronic nose, machine vision, differential scanning calorimetry (DSC), nuclear magnetic resonance (NMR), texture analyzer, real-time PCR, and DNA- and protein based methods, among others, are increasingly used for safety and quality assessments (Chapter 2). DNA-based techniques are used for identificatio of fis species. Marine resources provide rich sources of nutraceuticals and functional food ingredients. These ingredients belong to a wide range of chemical compounds with beneficia health effects. Use of marine oils in pharmaceuticals and some of the other marine-based products for health promotion and disease risk reduction is now common place and further progress in these areas is expected.

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Part I
Seafood quality

2 Practical evaluation of fish quality by objective, subjective, and statistical testing

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2.1 Introduction

The practical evaluation of seafood quality has to be considered on a global basis. The consolidation of global retail chains leading to uneven bargaining has resulted in lower profi margins for their suppliers; while globalisation has also driven the requirement for greater standards on residues and traceability. It is imperative that, to ensure consumer confidence validated traceability systems are introduced that can trace back any feature from source to consumer. In addition, consumers desire more information about the seafood products they buy. They want to know where the product is caught/farmed, how it is caught, and the environmental issues concerning sustainability of fis stocks, etc. Some large retailers are developing policies that demand supplies only from sustainable sources and the use of an eco-label to differentiate products. Eco-labels, such as the Marine Stewardship Council (MSC) for fis stock sustainability, is a policy to generate a market-based approach to address environmental issues. However, seafood eco-labels as a discriminating factor in consumer choice is uncertain and buying cues seem to be more dependent on other attributes such as quality and price [1].

Of fisher production, 40% is internationally traded, with 80% of all fis being consumed by Japan, the EU, and the US. The composition of this trade is changing from bulk commodities towards expanded trade in fresh products and especially processed consumer goods. This has led to greater challenges with regards to sanitation and quality procedures, which are more difficul to achieve in the developing countries [2]. Therefore, the drive for further scrutiny of the supply chain will inevitably lead to a greater demand for reliable and rapid quality testing.

All trade in fis procurement proceeds via purchase specifications The purchase specifi cation is a contract of quality that the supplier has to adhere to. The UK Sea Fish Industry Authorities have developed guidelines for fis purchase specification [3]. These guidelines make extensive use of objective sensory fis quality assessment systems evaluating morphological as well as sensory characteristics of the whole fish However, the guidelines do not recommend any additional instrumental testing to ensure the quality of the product, and this probably reflect the cost of instrumental testing that is likely to be too expensive for small trading units. However, the trend towards trade of more processed fis products (which

ultimately leads to the redundancy of raw whole fis evaluation procedures) and the demand from supermarkets, for greater quality and traceability, may change this perspective.

The ultimate goal of the fis industry is the purchase and favourable consumer perception of the seafood products on the market. Much of this favourable response will be achieved by providing a product at the right price with high eating quality. To achieve this goal, a practical approach to much of the research as discussed in this book has to be implemented while understanding the needs and demands of the consumer. Therefore, this chapter reviews the most up-to-date information available on quality indices from both consumer and industrial perspective for seafoods. It also examines the ways that future technology can meet the needs of consumer demands for quality.

2.2 Methods used for fish freshness and quality assessment: from source to the consumer

Freshness is the major contribution to the quality of seafood products. For all kinds of seafood products, freshness is essential for the quality of the fina product. It has been frequently stated that no single method is reliable enough for assessment of freshness and quality of seafood products. Therefore, a range of subjective (sensory), objective (non-sensory), and statistical methods have been proposed for evaluation of freshness and quality (Fig. 2.1). Each method has its particular advantages and disadvantages.

2.2.1 Latest developments in sensory methods

Considering all the developments in instrumental methods that have occurred in the last decade [4,5], sensory methods remain the most satisfactory way of assessing the freshness of fis and fisher products. Objective seafood sensory tests, based on certain attributes of raw fis (skin, eyes, gills, texture, etc.) [5–9], are the most commonly used methods for quality assessment of raw whole fis in the inspection service and fishin industry. However, these schemes are not universal in their application and modification are required to improve the accuracy for each seafood species studied. For example, Sveinsdottir *et al.* [10] developed the Quality Index Method (QIM) scheme to be used for Atlantic salmon. QIM has been recommended for a European initiative to harmonise this type of measurement, and QIM schemes have been developed for various common European fis species. However, further research is needed to evaluate the applicability of QIM for fis handled, stored, and processed under different conditions. A further interesting initiative is the development of software for QIM using a convenient hand-held terminal that allows data to be electronically imputted during sensory assessment [5,11].

Processing of fis leads to more challenging issues for the sensory specialist. The freshness of raw fillet are generally evaluated on colour and smell. It is more common to cook fillet before carrying out sensory evaluation and the Torry scheme has been developed to evaluate freshness of cooked fillet [12]. However, sensory assessment and measurements are not always well documented. Many companies have their own sensory schemes that have been developed for specifi purposes. In all cases, objective sensory measurement requires panel training or the use of experts. Panel training improves the reproducibility of sensory data and, therefore, ensures more coherent sensory testing. However, research has shown that panel training does not improve the threshold levels of panellists, but instead improves their

FISH FRESHNESS/QUALITY ASSESSMENT

SUBJECTIVE (*SENSORY*) METHODS

OBJECTIVE (*NON-SENSORY*) METHODS

STATISTICAL METHODS

SENSORY SCORING & GRADING
Torry Sensory Scheme
Tasmanian Food Research Unit (TFRU) Scheme or
Quality Index Method (QIM)
European Union (EU) Grading Scheme
Flavour Profile Analysis (FPA)
Artificial Quality Index (AQI)

CHEMOMETRICS/SENSOMETRICS
Partial Least Squares Regression (PLSR)
Principal Component Analysis (PCA)

MICROBIOLOGICAL/BIOLOGICAL
Total Viable Count (TVC)
Specific Spoilage Organism (SSO)
API Test Kits
Gram-Negative and Gram-Positive Microplates
Microbial Identification Inc. (MIDI)
Fatty Acid Methyl Ester (FAME)
Bacterial Biosensors
Polymerase Chain Reaction (PCR)
Quantitative PCR (qPCR)
Ribotyping
Oligonucleotide Probes
Predictive Food Micromodel (PFM)

PHYSICO-CHEMICAL
Freshness Meters (Torrymeter, Rtmeter, Intellectron
Fischtester VI, Freshness Grader & pH-meter)
Colour (Colour Meter & Spectropen)
Texture (Texture Analyser & Hand-Held)
Electronic Nose (LibraNose & FreshSence)
Electronic Tongue
Image Analysis/Machine Vision
Raman Spectroscopy & UV-Visible Spectroscopy
Near-and Mid-Infrared (NIR & MIR)
Electrochemical Impedance Spectroscopy (EIS)
Nuclear Magnetic Resonance (NMR)
Differential Scanning Calorimetry (DSC)
Time Domain Reflectometer (TDR)
Two-Dimensional Gel Electrophoresis (2DE)

CHEMICAL/BIOCHEMICAL
Adenosine-5'-Triphosphate (ATP) Breakdown
Compounds
K and Related Values (Ki, G, P, H & Fr)
Biogenic Amines
Volatile Compounds
Trimethylamine (TMA)
Dimethylamine (DMA)
Total Volatile Base-Nitrogen (TVB-N)
Thiobarbituric Acid-Reactive Substances (TBARS)
Formaldehyde
Ammonia
Indole
TOTOX [2xPeroxide Value (PV)+*p*-Anasidine(*p*AV)]
Conjugated Diens (CD)

Fig. 2.1　Methods used for fish freshness and quality assessment.

discriminatory ability and quality of vocabulary used. Training of panellists is therefore highly specialised, with panellists findin it hard to use their increased sensory knowledge on other similar products (e.g. the acuity of people's perception is not on a physical but on a cognitive level) [13]. Furthermore, training of a panel of experts is expensive and time consuming and therefore out of reach of most small- to medium-sized (SME) companies. Nevertheless, recommendations for panel training range from 10 to 120 hours and there seems a good correlation between panel training and panel performance [14]. There has also been work carried out in generating guidelines for the accreditation and proficien y of sensory testing, and the quality of sensory data is likely to improve with the implementation of these guidelines [15].

2.2.2   Latest developments in non-sensory methods

Sensory methods such as the QIM scheme are non-invasive and are clearly favoured by regulatory bodies for analyzing raw whole fish However, processing removes morphological characteristics that lead to alternative non-sensory instrumental methods (e.g. chemical/biochemical, physico-chemical, and microbiological/biological) becoming increasingly more favourable (Fig. 2.1).

2.2.2.1   *Chemical/biochemical methods*

Chemical/biochemical techniques such as adenosine $5'$-triphosphate (ATP) breakdown compounds, K and related values, trimethylamine (TMA), total volatile base-nitrogen (TVB-N), thiobarbituric acid-reactive substances (TBARS), and biogenic amines, etc., remain to be used for assessing fis freshness [4].

Dynamic headspace analyser-gas chromatography-mass spectrometry (DHA-GC-MS), static headspace analyser-gas chromatography-mass spectrometry (SHA-GC-MS), gas chromatography-olfactometry (GC-O), solid-phase microextraction (SPME), and electronic nose, etc. have been successfully used to measure the specifi odours produced by seafoods [16,17]. Much effective work has been carried out to evaluate the odour using these techniques, mainly focusing on developing a workable model to predict the sensory response [18,19]. However, more interestingly, volatiles analyzed by these chromatographic techniques have also shown to be better predictors of shelf-life than total viable counts (TVC) in cold smoked salmon [20]. Although these techniques have the potential as powerful predictors of fis quality, with the need to purchase and maintain this complex and expensive equipment, their widespread practical application beyond research has not been as widespread as would be expected.

2.2.2.2   *Physico-chemical methods*

As shown in Fig. 2.1, several new and rapid physico-chemical methods have been successfully employed by the seafood industry. Instrumental techniques using ultraviolet-visible (UV-VIS) spectroscopy [21,22], near-infrared (NIR) [22,23], mid-infrared (MIR) [24], electronic nose [17,25], image analysis [26], colour [27], differential scanning calorimetry (DSC) [28], texture analyzer [29], and nuclear magnetic resonance (NMR) [30] have the advantage of being non-invasive and rapid, enabling them to be potentially used in an on-line situation. Spectroscopic techniques such as UV-VIS, NIR-MIR, and NMR provide varying degrees

of information regarding the chemical composition of the product in a rapid non-invasive analysis.

Additional instrumental developments, with the potential for rapid non-invasive analytical quality measurements, have been reported recently in the literature and include Raman spectroscopy [31,32], dielectric measurement [33,34], electronic tongue (potentiometric electrodes) [25,35,36], and two-dimensional gel electrophoresis (2DE) [37]. Raman spectroscopy is a good technique to investigate modification in lipids, protein structure, and water in muscle foods. Therefore, this technique has been used to quantify protein solubility, water holding capacity (WHC), peroxide value (PV), and fatty acid composition in fis [31]. Correlation studies between PV and Raman data have also been carried out in mackerel. Dielectric measurements and time domain reflectometr (TDR) from the surface of fis samples were used to examine the quality of Baltic cod. Multivariate statistical techniques were used to correlate QIM with instrumental data. A reasonable estimate of fis freshness was obtained when using TDR; however, if the data were combined with the output from an electronic nose, the predictability of the model improved [33]. Two recent excellent chapters on time domain spectroscopy [38] and measuring electrical properties, such as Fischtester and Torrymeter [34], have been published.

Gil *et al.* [36] have reported the development of an electronic tongue (an electronic tongue makes contact with the analyte, whereas the electronic nose is dependent on the analysis of the headspace above the analyte), which was comprised of 16 potentiometric electrodes of the metal, metal oxide, and insoluble metal salt type built with thick fil technology. Multivariate statistical techniques were used to correlate the sensor outputs with chemical measurements of freshness in sea bream, including biogenic amines, pH, microbial analysis, and TVB-N. Good correlation coefficient ($r^2 > 0.95$) were found using a fold training/validation approach [36]. The same research group also found good correlations using simple linear regression between the outputs of Ag and Au electrodes and the K_1 index (Eqn. 2.1). The theoretical reason for this correlation was discussed [35]:

$$K_1 = 47.58 - 46.64. \ Ag - 597.96. \ Au \ (r^2 = 0.96) \tag{2.1}$$

One interesting approach to overcome the disadvantages of each rapid technique for investigating quality has been considered by Olafsdóttir *et al.* [39]. The authors recognised that each physico-chemical technique was particularly useful at measuring certain quality attributes (e.g. electronic nose for odour analysis and texture analyzer for texture analysis). They have proposed a multisensory approach, where combining the output of complimentary sensors can improve the correlation with fis freshness (Fig. 2.2). In fact, it has been claimed that the Artificia Quality Index (AQI), which is the combined data of the instrumental techniques, predicts the freshness of the fis as well as the QIM [40].

2.2.2.3 *Microbiological/biological methods*

To develop a totally satisfactory model using microbial testing, a thorough understanding of the nature of the bacterial flor and how it varies needs to be established. Bacterial contamination from the intestinal tract, skin, and gills occurs after death and the microflor that dominates is dependent on the natural habitat (surrounding water, temperature, feeding habits, etc.), storage conditions, and other potential contamination routes after the catch. For example, initially psychrophiles will be present in large numbers of fis from temperate climates, whereas mesophiles are liable to dominate in fis from tropical climates. However,
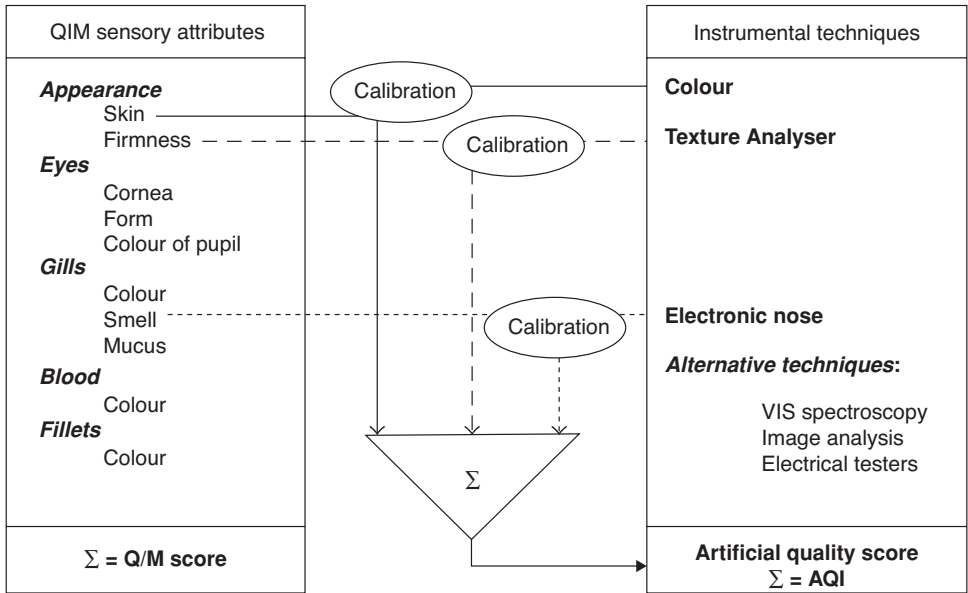
Fig. 2.2 Construction of the AQI. After calibration with sensory data (QIM) the instrumental readings are combined into artificial quality score giving the AQI. Adapted with permission from Di Natale [82], with permission of Wegeningen Academic Publishers.

during chilled storage, psychrotrophs will be selected. Conventional and rapid microbiological methods used for fis freshness and quality assessments as well as bacterial identificatio are given in Fig. 2.1.

Standard guidelines for acceptable levels of fis freshness use total viable counts (TVC) or aerobic plate counts (APC) with levels of 10^6 cfu/g being considered unacceptable. These methods have been used extensively throughout the world, although Olafsdóttir *et al.* [41] reports that fis TVCs are somewhat higher at 10^7–10^8 cfu/g at the point of sensory rejection. In relation to the EU-project (FLAIR-FLOW EUROPE) [42], TVC levels in seafood correlate poorly with the degree of freshness or remaining shelf-life (Fig. 2.3). During the storage of seafood at particular conditions of temperature, atmosphere, percent salt, water activity, and preservatives, etc., specifi spoilage organisms (SSO) grow faster than the remaining seafood microflor and eventually produce the metabolites responsible for off-fl vours and sensory product rejection. Consequently, the number of SSO and the concentration of their metabolites can be used as objective quality indices for shelf-life determination in seafoods [42]. By definition SSO levels should correlate well with seafood freshness [43,44] and search for SSO has led to the development of microbial tests for *Sherwanella putrefaciens* (hydrogen sulphide producer), *Photobacerium phosphereum* (important in some modifie atmosphere packed fish) and *Pseudomonas* spp. (important for marine, temperate, fresh fis stored aerobically in chilled conditions). Good reproducible and repeatable data may be generated from valid microbial growth techniques. However, these techniques are generally slow, requiring a period of incubation lasting up to 10 hours to 5 days. This has led to the development of new and rapid microbial analytical techniques/methods (non-molecular and molecular), which have been successfully used in the seafood industry within a working day or less (Chapter 19). Some of the non-molecular techniques include API test kits [45], Gram-negative and Gram-positive microplates [45–47], Microbial Identificatio Inc. (MIDI)
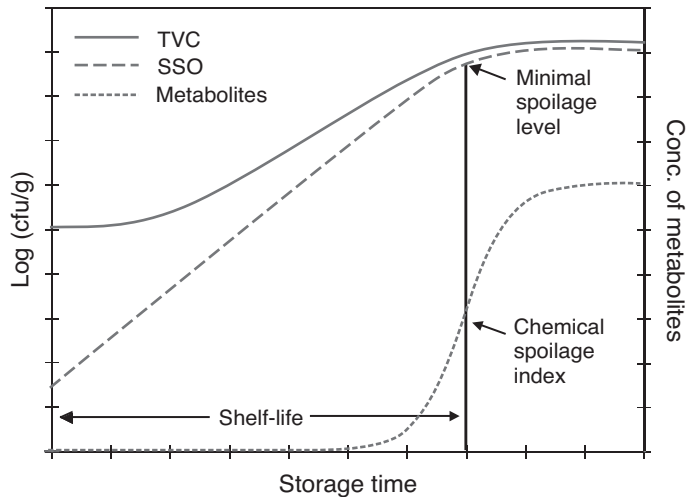
Fig. 2.3 Typical changes in TVC, SSO, and metabolites produced by SSO during the storage of fresh seafood. Adapted from Dalgaard [42], with permission of TEAGASC.

[48,49], and fatty acid methyl esters (FAME) [46,50]. Polymerase chain reaction (PCR) and its application, quantitative PCR (qPCR), ribotyping, oligonucleotide probes, etc. are some examples of molecular techniques [42,51–58].

It is intuitive to consider that seafood freshness is correlated to the microbial profile and the development of robust computer based mathematical models such as the predictive food micromodel (PFM) have been useful tools to predict microbial numbers and remaining shelf-life in seafood [59]. However, each species of seafood needs to be considered separately as to which process dominates in determining shelf-life. For example, in cephalopods it has been reported that endogenous enzymes were the main systems responsible for changes in sensory attributes [60] with microbial loads being of secondary importance for early shelf-life prediction.

2.2.3   Latest developments in statistical methods

Much of the instrumental and sensory data used for assessing fish quality have been used in predictive instrumental studies. The ultimate goal of these studies is to assess the consumer response via an instrumental technique in real time. To obtain a good model, valid data must be collected at various points and appropriate valid statistical techniques need to be used. These predictive instrumental studies are, therefore, heavily dependent on the use of appropriate statistical techniques to generate satisfactory insightful conclusions.

Spectroscopic analysis correlated to individual quality attributes, for example, white spot formation during frozen storage of shrimp, does not require complicated statistical interpretation of the data. As in this case, spectral results are specifically related to calcium carbonate crystal formation, which is accompanied with identifiable spectral bands [61]. Nevertheless, simple linear regression is unlikely to be successful for evaluating complex quality attributes such as flavour, and multiple regressions can result in multicolinearity leading to unreliable models. Stepwise regression may overcome multicolinearity to a

certain degree, but then useful information is lost for which attributes are correlated. Multivariate statistical techniques are able to help overcome these problems.

The two major multivariate statistical techniques used are principal component regression (PCR) and partial least squares regression (PLSR). PCR is a "one data block" method that examines correlations in the multidimensional data block and then applies a linear transformation to the data block to summarise the correlated variables by a reduced number of components (the principal components). These principal components explain a fraction of the variability in the data block and can be visually presented (principal component analysis (PCA). The principal components can then be considered the predictor variables for a regressional analysis of the "y" predicted variable data block (PCR). In contrast, PLSR is a "two data block method", where factors are derived from both the predictor variable data set and the predicted variable data set. Each factor identifie by PLS (unlike PCA) has been derived to ensure maximum predictive power and seems to be the preferred method of choice for developing models.

Downey [23] used NIR and a fibr optic probe to measure non-invasively the moisture and oil content in salmon. Stepwise multiple regression, PCR, and PLS were performed on the data set. PLS was, without exception, the best mathematical procedure for the prediction of moisture and oil in salmon [23]. A similar result was also observed for predicting QIM scores using visible spectroscopy, where PCA was used to look for diversity among the spectra, and PLS was used to carry out the multivariate regression. Using PLS, VIS spectrophotometry was able to predict QIM scores, and the authors considered the method robust enough for commercial use [21].

More detailed structural information is obtained by Fourier transform-mid infrared (FT-MIR) (Fig. 2.4), which is more easily correlated to specifi chemical components (e.g. sugars). However, univariate data analysis seems to be inappropriate for the analysis of a complex commodity such as seafood, and the use of multivariate statistics seems to provide
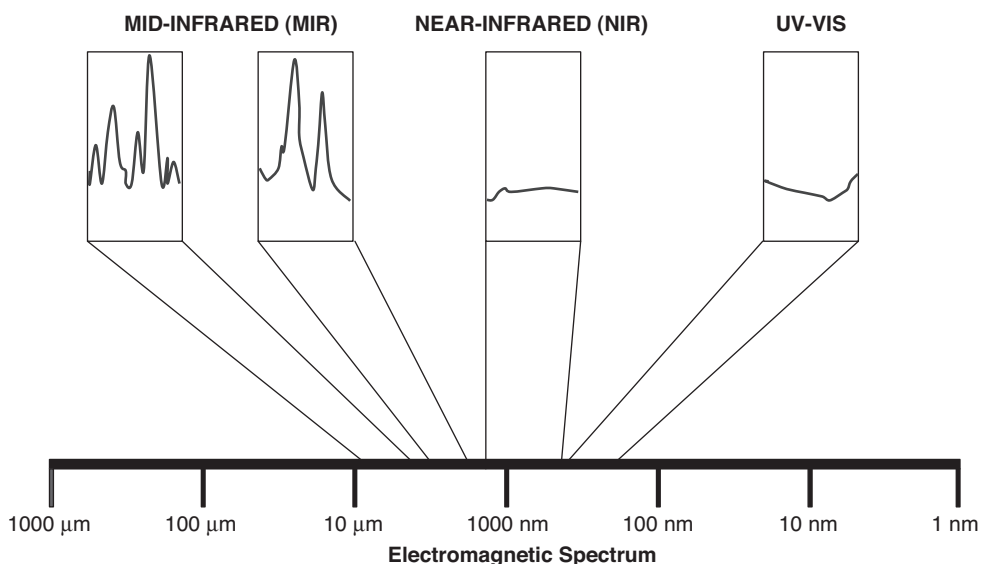
Fig. 2.4   A representation of the type of absorbance peaks that are found for VIS, NIR, and MIR spectroscopy for seafood samples.

the only solution when evaluating quality of seafood. For example, Karoui *et al*. [24] reported using PCA followed by factorial discriminate analysis (FDA) on the firs fi e principal components to help develop a model using FT-MIR to differentiate fresh and frozen-thawed whiting fillets

Morita *et al*. [19] compared descriptive sensory attributes with that of GC volatile profile of fis broths prepared from 16 fis species. They used PCA and cluster analysis to classify the fis broths into four groups characterised by their sensory profile They then used PLS to identify "influentia peaks", followed by PLSR to model these "influentia volatiles" with the sensory attributes as described by a trained panel. They found that 20 "influentia volatiles" were highly predictive of sensory characteristics, as described by 14 screened and trained panellists. Similar work was carried out by the same authors on boiled squid, prawn, and scallop [18]. One of the major criticisms of PLSR and PCR analysis of GC-MS volatiles with sensory characteristics (as described by a trained panel) is that the relationship between dependent and independent variables (e.g. sensory characteristic response with volatile concentration) may not be causal. Therefore, statistical techniques need to be considered only as a tool to examine possible relationships. To develop validity in this approach and to establish a strong robust model, the odour and more preferentially the aroma profil of the influentia volatiles examined and their perceptual blend should be considered [62]. Furthermore, careful consideration should be given to how volatiles are extracted and how closely this resembles the eating process. Retronasal studies seem to indicate that the physiology and psychology of aroma perception is a complex process, where intra-individual and inter-individual differences should not be ignored when developing an instrumental model to predict aroma perception [63].

2.2.4   Consumer testing for fish quality perception

Much work has been carried out in examining consumer motivation to purchasing seafood; however, in contrast, research on consumers' fis quality perception is more limited, but still important [64]. However, this gap in understanding has recently been redressed. Cross-sectional data was collected from a sample of 429 consumers in Belgium. The authors suggested that two dimensions shape fis quality evaluation; the importance that people attach to quality and the confidenc in making a decision about the quality of the product. Hierarchical clustering was performed on the data, which allowed identificatio of four fis consumer segments in relation to the two identifie perceptions. These clusters were labelled as "uninvolved, uncertain, self-confident and connoisseurs" [64]. These four consumer segments are reported to have differing values. For example, the consumer segment labelled "uncertain" and "connoisseurs" report that they are more interested in fis quality labelling as a purchase cue, whereas it is more likely that the "uninvolved" consumer segment are unlikely to express their attitudes about quality but are likely to make decisions based on a quality indicator such as expiry date, price, and convenience [64].

One of the criticisms with regards to some consumer research is that in many cases conclusions are expanded beyond the consumer group that the work was initially carried out in. Therefore, this type of research needs to be expanded to examine markets both within Europe and on a worldwide basis.

Questionnaire surveys using hedonic scales seem to remain the preferred technique used by consumer researchers [1,65,66]. However, other consumer techniques can be employed. For example, we have used Q methodology to assess consumer perception of shellfis presentation in supermarkets. This technique shows promise as a tool for identifying consumer

values [67]. Fong and Anderson [68] used conjoint analysis to help study integrated shark fi market preferences. Nevertheless, techniques such as laddering, conjoint analysis, and repertory grids seem to be rarely used. There is a lot of very good consumer work that is carried out for or by companies but, unfortunately, proprietary information of this sensitivity is very rarely published.

With regards to expanding the seafood market in an environment of depleting natural fis stocks, the consumers' quality perception of aquaculture is probably more important. Again, the little research reported in this area seems to indicate is that consumers consider farmed fis as having a lower intrinsic quality and is one of the main reasons for them not purchasing farmed fis [69]. Verbeke *et al*. [66] studied the perception of Belgian consumers towards wild and farmed fis as obtained from a survey questionnaire. Consumers were asked about their perception of wild and cultured fish by comparing both types *via* six attributes, namely health, safety, taste, nutritional value, versatility, and availability. Mean perception scores were slightly in favour of wild fis on the attributes of taste, health, and nutritional value. In addition, the consumer perception of safety did not differ between the two groups.

2.3   Potential use of micro- and nanotechnologies

Micro- and nanotechnologies allow the creation of structures, devices, and systems that have a high density of features and therefore enhanced functionality. Nanotechnology is widely accepted as the ability to create and use features that are below 100 nm in one or more dimensions. Micro- and nanotechnologies are generating considerable excitement within the food sector because they offer the potential for radical innovation [70,71]. Specifi areas of innovation that use micro- and nanotechnologies include food safety and quality, as well as packaging [72–74]. Nanocomposites, a relatively new family of composites materials, are attracting considerable attention for packaging applications. The addition of low load-ings of nanoparticles can provide a significan enhancement in the properties of packaging materials, including mechanical, thermal conductivity, and gas barrier properties, without compromising other relevant attributes such as toughness and transparency [75]. Interesting new approaches also offer the potential to incorporate electronic and sensing functions on polymer materials to create intelligent packaging. Pacquit *et al*. [76] have developed a pH indicating sensor entrapped within a PET polymer matrix spun to an average thickness of 2.57 to 1.01 µm. This sensor showed interesting positive correlation with TVC and *Pseu-domonas* spp. in freshly caught whole cod (*Gadus morcua*) and whole whiting (*Merlangius merlangus*) during storage experiments.

Micro- and nanotechnology offer the potential of creating inexpensive, robust, and portable devices that can provide chemical and biological information that is difficul to obtain [77]. Miniaturised diagnostic systems are being developed that can carry out sophisticated biological and chemical processing. These systems handle small flui volumes and are sometimes referred to as microfluidi devices. Systems that are developed to perform a specifi series of operations for a particular application are known as lab-on-chip. The development of these types of systems requires the integration of a variety of disciplines, including micro- and nano-manufacturing, biology, materials, and electronics. Many of the manufacturing approaches are those borrowed from the semiconductor industry and, in an analogous manner to integrated circuits, they offer the potential for high functionality, reproducibility, and volume, as well as low cost [78].

These microfluidi devices are being developed to integrate a variety of analytical functions, such as sample separation and detection, and carry out sophisticated operations on small flui volumes in a very reproducible manner. The ability to perform operations on small flui volumes provides an advantage of reduction of cost of reagents, which can be high, particularly for biological analysis.

The design and manufacturing approach for miniaturised diagnostic systems is dependent on the application that will dictate the materials to be used. The materials that are commonly used include polymers, glass, silicon, or a hybrid of these materials. Polymers are ideal for low cost and single use devices that can be manufactured in large volumes. Glass has the advantage of beneficia material properties such as surface charges for the development of separation systems and transparency for optical detection. Silicon is ideal if there is a requirement to integrate electronic and fluidi functions. Silicon itself is not an ideal material for flui manipulations but its oxidised form, SiO_2, is a good insulator and can be used to protect the silicon from contact with solvents. Hybrid materials, such as silicon and glass, may offer advantages for specifi applications and should therefore also be considered.

Microfluidi devices require channels within the substrate through which the flui can be transported. A variety of approaches are used for creating this channel architecture. Polymer devices can be fabricated using laser ablation, hot embossing for higher volumes, and micro-injection moulding. Wet etching and/or powder blasting are preferred for glass based microfludi devices. Silicon based devices often employ semiconductor processes such as photolithography and offer the potential of very large numbers of devices with low overall cost but do require expensive capital infrastructure, such as a clean room and associated processing equipment, for device fabrication. The channels within the substrate need to be sealed with a lid to contain the flui and this can be carried out using various methods including solvent, adhesives, and ultrasonic bonding.

A number of different methods are available for transporting the flui through the device including centrifugal, electrokinetic, and pressure. These approaches have their advantages and disadvantages on application. Centrifugal approaches are implemented on compact disc (CD) based microfluidi devices. In this case, control of flui operations is dependent on the channel architecture on the CD as well as on the speed of rotation. This approach is attractive since it allows multiplexing, for example, a large number of analytical operations can be performed simultaneously in an easy manner. The drawback is that the flui operations are dependent on the properties of the flui and therefore it is difficul to develop a CD microfluidi device for a variety of applications using different samples types. Pressure driven approaches are relatively easy to implement, using, for example, a syringe with a driver, but are less attractive where large pressures are required to drive the fluid such as long and narrow channels, and/or where multiplexing is required. Electrokinetic approaches allow implementation of multiplexing as well as use in long and narrow channels but require the substrate material to have surface charges. This can be difficul when polymer substrates are used. A variety of other on-board flui driving systems is currently being investigated but is less close to commercialisation. A variety of transduction approaches can be implemented including optical and electrochemical methods. The transducer scheme to be implemented is dependent on the analysis to be performed. Electrochemical approaches are attractive since they can be integrated as part of a microfluidi device using conventional semi-conductor type processes. The integration of optical elements as part of the microfluidi device is more difficult Optical detection schemes that rely on absorption face difficultie of limited sensitivity due to the small channel path length.

Seafood developments using this technology include the application of a lab-on-chip device for rapid analysis of biogenic amines. This microchip device performs both precolumn derivatisation and electrophorectic analysis with claimed detection limits down to 1 nm and total analysis time of less than 60 seconds [79]. Validity of the technique was demonstrated on a Thai fis sauce sample where putresine and histamine were identified Fish species identificatio has also employed lab-on-chip technology [80], and also a microfluidi device for the detection of a marine fis iridovirus has been developed, using enzyme-linked immunosorbent assay (ELISA) with quantum dots as the labelling flurophor [81].

Much work is being carried out in the development of micro- and nanosystems for different sectors. However, there is a need to bridge the gap between this emerging fiel and the food industry to realise the wide scale application of this technology.

2.4   Conclusions

The need for rapid analytical techniques to measure seafood quality and freshness is greater than ever. Consumer studies appear to indicate that quality is still the key buying cue for fis purchases. Sensory evaluation is considered the most effective technique to measure fis quality; however, developments in instrumentation, understanding, and statistical techniques have created a number of promising new opportunities. More focus on underpinning science to ensure the stability of the models proposed by complex statistical techniques may enable a rapid increase in the potential use of this technology by demonstrating repeatability and reproducibility to the seafood industry. The emerging fiel of nanotechnology has the potential to revolutionise the whole seafood supply chain.

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3 Sensory evaluation of fish freshness and eating qualities

David P. Green

3.1 Introduction

Sensory evaluation of fis freshness and eating qualities remains the most important assessment method employed by the seafood industry today. Until recently, sensory analysis was used to determine product specification or standards in quality control and to an extent, in product development and optimization [1]. However, sensory assessments are becoming increasingly important in market development and frequently are correlated with other non-sensory chemical, microbial, and physical assessment techniques (Chapter 2).

Regardless of the species, consumers generally believe that the best fis is the freshest fish and thus are more desirable. A widely accepted definitio for fis freshness is difficul however, because from a species standpoint, freshness is constantly changing and forms a continuum from the fresh to a stale state in terms of its eating qualities. Freshness makes a major contribution to the overall quality of fis and fisher products [2] and is greatly influence by both pre-harvest conditions and post-harvest handling practices [1,3].

Bremner and Sakaguchi [4] firs described freshness as a total set of characteristics in recently harvested products that bear on its ability to meet stated or implied requirements. As fis spoils, its smell, taste, appearance, and texture go through characteristic and well define stages. Sensory assessors are trained to recognize these stages in going from fresh to stale and assign an objective number or appropriate score to each fis or batch of fish Recognition of freshness and the acceptance or rejection of fis on this basis may be all that is necessary for industry. However, different species and products spoil in different patterns and the use of appropriate sensory and non-sensory assessment methods is warranted.

Bremner [5] also recognized that the term "eating qualities" should be replaced by a more direct expression of the properties or group of properties that best describes the food product. Nielsen *et al*. [6] point out that sensory evaluation mostly describes intrinsic product qualities (i.e. species, fat content, smell, and appearance) while consumer choice is based on both intrinsic and extrinsic factors (i.e. price, convenience, origin, and handling). The validation of intrinsic sensory tests (descriptive/discriminative – objective methods) for use in predicting the remaining shelf-life of fis in ice has been reported [7]. Future integration in the assessment of intrinsic qualities by sensory tests and extrinsic factors (preference/acceptability –

subjective methods) may soon be used in predicting consumer behaviour towards a particular fis product [8,9].

Consumer demand for high-quality, safe, and healthy foods is increasing on a global basis. Improved freshness tests have been developed for various fis species that are both rapid and more reliable. The use of sensory assessments in fis is becoming increasingly important for new market development. This chapter discusses the sensory methods used for evaluating fis freshness and eating qualities and some pre- and post-harvest practices that affect the quality and shelf-life of chill-stored fish

3.2 Methods for sensory evaluation of fish

Both objective and subjective sensory testing methods are used to evaluate fis freshness. Objective tests include descriptive (profilin and structured scaling) and discriminative (triangle test and forced choice) sensory methods. Both descriptive/discriminative tests are analytical measurements of the intrinsic quality of the product, whereas effective (subjective test) methods are used for consumer testing and measure the attitude and emotional responses of consumers towards a particular product [8]. For chill-stored fish sensory methods based on the Torry scale [10] remain in use for some countries. In Europe, the most common method used for quality assessment of chill-stored fis is the EU scheme [11]. Most recently, a structured scaling and sensory profilin method (Quality Index Method [QIM]) was developed [12] to assess fis freshness [8]. The latter method is thought to address some limitations inherent in the current EU grading scheme.

3.2.1 Torry scale

The firs scoring method for use with fis and fisher products was developed at the Torry Research Station in the UK [13]. The Torry scale is a 10-point scale originally developed to assess the eating qualities of cooked fis samples. Scores are given from 10 (very fresh in taste and odour) to 3 (spoiled). Scores below a 3 are considered unnecessary, as the fis is then not fi for human consumption. The average score of 5.5 may be used as the limit for consumption. The Torry scale has been developed for lean, medium fat, and fatty fis species. The spoilage attributes can be observed in either the raw or the cooked fish and appropriate scoring systems are available for both forms (Table 3.1). Interestingly, the Torry scoring method for fis freshness was correlated with changes in electrical conductivity across skin surfaces of fish These relationships lead to development of the Torry Fish Freshness Meter (Torrymeter), which is commercially available and used today [14].

3.2.2 European Union Scheme

Quality assessment of chill-stored fis under Council Regulation (EC) No. 2406/96 November 26, 1996 established the EU scheme used by fis inspectors today [11]. The scheme identifie three quality levels: E (Extra) is the highest quality; A is acceptable quality; and B is the level beyond which fis are not admitted for human consumption (Table 3.2). The EU scheme is criticized for its limitations in that it does not take into account the differences between species (uses only general parameters) and mixes both subjective and objective sensory methods in the assessment scheme. Several studies have shown that the more recent QIM scheme is more reliable in assessing the sensory changes of different species as compared to the EU grading scheme [15].

Table 3.1 Torry score sheet for cod (cooked) from gutted fish chilled in melting ice. Adapted from Hyldig et al. [1]

Score	Odour	Flavour	Texture, mouth feel, and appearance	Score
10	Initially weak odour of sweet, boiled milk, starchy, followed by strengthening of these odours	Watery, metallic, starchy; initially no sweetness but meaty flavours with slight sweetness may develop	Dry, crumbly with short tough fibres	10
9	Shellfish, seaweed, boiled meat, raw green plant	Sweet, meaty, creamy, green plant, characteristic		9
8	Loss of odour, neutral odour	Sweet and characteristic flavours but reduced in intensity	Succulent, fibrous; initially firm going softer with storage; appearance originally white and opaque going yellowish and waxy on storage	8
7	Wood shavings, wood sap, vanillin	Neutral		7
6	Condensed milk, caramel, toffee-like	Insipid		6
5	Milk jug odours, boiled potato, boiled clothes-like	Slight sourness, trace of "off" flavours		5
4	Lactic acid, sour milk, "byre-like"	Slight bitterness, sour, "off" flavours		4
3	Lower fatty acids (e.g. acetic or butyric acids), composted grass, soapy, turnipy, tallowy	Strong bitter, rubber, slight sulphide		3

Table 3.2 EU freshness scheme for white fish (cod, haddock, saithe, pollock, whiting, plaice, redfish, and hake). Adapted from Hyldig et al. [1]

Criteria	Freshness category			
	Extra	**A**	**B**	**Not admitted**
Skin	Bright, iridescent pigment, no discolouration	Pigmentation bright but not lustrous	Pigmentation becoming discoloured and dull	Dull pigmentation
Skin mucus	Aqueous, transparent	Slight cloudy	Milky	Yellowish, grey, opaque mucus
Eyes	Convex, black, bright pupil, transparent cornea	Convex/slightly sunken, black, dull pupil, slightly opalescent cornea	Flat, opalescent cornea, opaque pupil	Concave in centre, grey pupil, milky cornea
Gills	Bright colour, no mucous	Less coloured, transparent mucus	Brown/green discolored, thick, opaque mucus	Yellowish, milky mucus
Peritoneum on gutted fish	Smooth, bright, difficult to detach from flesh	Slightly dull, can be detached from flesh	Speckled, comes away easily from flesh	Does not stick
Smell of gills	Seaweedy	No seaweed smell, neutral Oily, seaweedy or slightly sweetish	Fermented, slightly sour	Sour
Abdominal cavity	Oily, peppery, earthy smell		Oily, fermented, slightly rancid	Sour
Flesh	Firm and elastic, smooth surface	Less elastic	Slightly soft, less elastic, waxy and dull	Soft, scales easily detach from skin, surface wrinkled

3.2.3 Quality Index Method

The QIM was developed at the Tasmanian Food Research Unit (TFRU) of the Commonwealth Scientifi and Industrial Research Organization (CSIRO), Australia in the late 1970s and early 1980s. The QIM scheme addresses some of the inherent limitations contained in the EU grading scheme [16,17]. Criteria include not only accuracy, precision, and robustness among different user groups but its adaptability to changing circumstances in meeting future requirements. Its ease of use, cost, and likelihood of adoption in various countries are other important features of the method [18]. The terminology used in developing the scheme is consistent with the concerns raised by Bremner and Sakaguchi [4]. It does not measure quality itself or freshness but rather the degree or rate of change in important criteria used to describe these qualities. The sum total of these changes can then be interpreted into equivalent days of storage and remaining shelf-life [7].

The QIM is rapidly being adopted in different countries as an alternate sensory technique using specifi descriptive attributes for selected species that are evaluated in sequence. Since all fis have their own distinctive spoilage patterns and sensory attributes, QIM schemes are developed for individual species. Each attribute is scored from 0 to 3 by novice or experienced assessors with low scores indicating the best quality. The sum of all attributes is called demerit points, or QIM index points. This value increases linearly with storage time in ice of a given fish Using the QIM system, the linear relationship between the quality index (QI) and storage time on ice, makes it easy to calculate the remaining shelf-life of fis   (Table 3.3) [19].

Table 3.3 Quality Index Method (QIM) scheme for hybrid striped bass (*Morone saxatalis* × *Morone chrysops*). Adapted from Nielsen & Green [19]

Quality parameters		Descriptions	Point
Whole fish	Skin colour/appearance	Pearl-shiny, iridescent pigmentation all over	0
		Less pearl-shiny, yellowish, stripes still distinct	1
	Odour	Neutral, pond, fresh fish, seaweed	0
		Melon, cucumber, green grass	1
		Cardboard, fishy, putrid, rotten	2
	Texture	In rigor	0
		Firm, resilient, finger mark disappears immediately	1
		Soft, finger mark still persists after 3 seconds	2
Eyes	Pupil	Black, clear, bright, iridescent	0
		Dark gray, meat, dull	1
		Milky, cloudy, hazy, light, gray	2
	Shape	Convex, bulging	0
		Flat	1
		Concave, sunken	2
Gills	Mucus	Transparent, clear, none	0
		Milky, clotted	1
	Colour/appearance	Bright red, red, burgundy	0
		Pale red, pink, light brown	1
		Brown, dull	2
	Odour	Pond, fresh fish, fresh rain	0
		Melon, cucumber, metallic	1
		Musty, fishy, putrid, rotten	2
Quality index (total score)			**0–14**

The principle behind development of the QIM scheme is summarized by Hyldig *et al*. [18] and specifi steps in development of schemes is provided in Bonilla *et al*. [7]. Training of assessors is performed for the industry and was adapted for use with consumers [20]. The consumer (C) QIM method uses an external consumer panel and a vocabulary (descriptors) specificall chosen for consumers. C-QIM is not an acceptability (subjective) test but an objective tool for use in decision making for the consumer when buying fis in a market or retail outlet [21]. Further work in this area is ongoing as new tools are being investigated for delivery of the QIM with new species and wider use by industry and consumer groups. Adoption of QIM in the EU and other countries would greatly aid in standardizing trade in fis and fisher products worldwide.

3.3 Pre-harvest factors affecting freshness

Deteriorative changes in fis muscle start at the point of death and are impacted by pre-harvest conditions that exhaust energy reserves in the fis [22]. The most dramatic change is the onset of rigor. At point of death, fis muscle is completely relaxed with an elastic texture that may persist for a period of time before the muscles begins to contract. When muscles become rigid, the whole body becomes infl xible and the fis enters rigor mortis, which can last for hours until the stiffness is resolved by the action of natural endogenous proteases (e.g. cathepsins, calpains, and collagenases) that attack various structural muscle proteins during post-mortem. Resolution of rigor mortis makes the muscle relax again and become limp. However, the muscle is no longer as elastic as before rigor. Typically, rigor mortis starts in vertebrate fis in 8 to 24 hours after death, but this period can be shorter or longer, and rigor is resolved after a further 1 to 3 days. Time into, time in, and strength of rigor varies with several factors (Fig. 3.1) including species, nutritional status, amount of exercise before death, and ambient temperature of the fish

Temperature remains the principal control measure to preserve fis quality. Rapid chilling at the point of harvest can significantl improve the shelf-life of fresh fish Delaying the onset of autolytic reactions and growth of micro-organisms associated with fresh fis gives the greatest extension in shelf-life of chill-stored fis (Fig. 3.2). "Rested harvest", a concept developed at the Plant and Food Research Institute in New Zealand, is based on this principle. Food grade anesthetics (Aqui-S^{TM}) reduce the stress on fis at time of harvest [23]. Rested harvest drops exhaustion of energy reserves in live fish delays onset of rigor development, and extends the amount of time fis are in rigor by several days. The optimal eating quality of fresh fis on ice occurs about two days after resolution of rigor and is closely related to temporary build-up of inosine monophosphate (IMP) in muscle tissues (Fig. 3.3). Delaying onset and extending the time fis muscle is in rigor effectively preserves quality and extends fresh shelf-life.

3.4 Post-harvest factors affecting freshness

Bleeding fis prior to or at the point of death is another effective technique to preserve freshness. The "ikejime method" is used for spiking large tuna to destroy the neural network between the brain and spinal column. In combination with temperature, this can be very effective in reducing discolouration and development of off-fl vours. High pressure processing and modifie atmospheric packaging (MAP) are other techniques used to reduce enzymatic
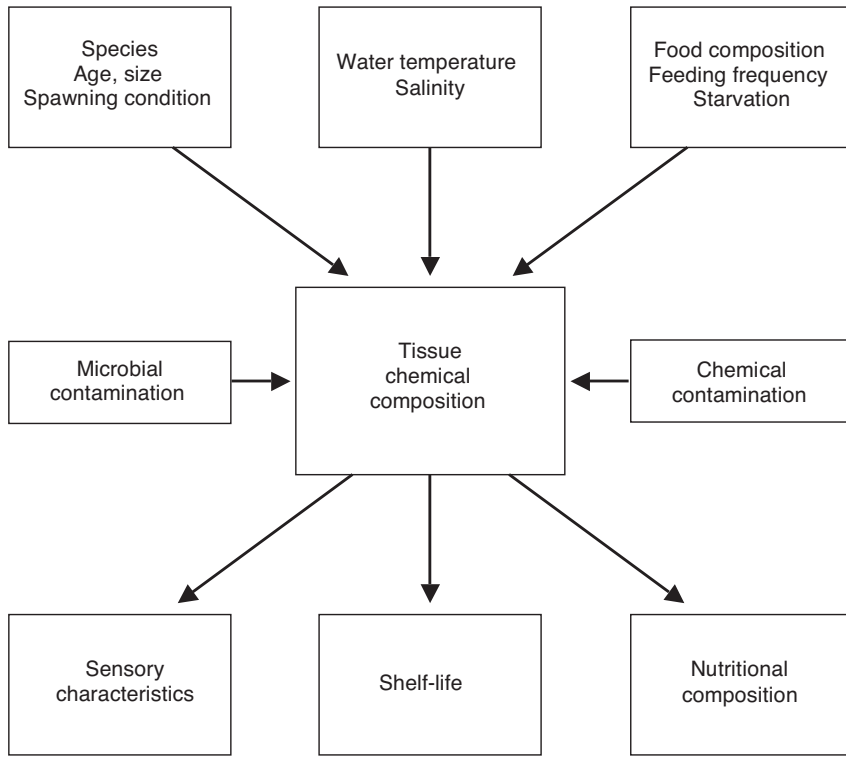
Fig. 3.1   Pre-harvest conditions that affect fish freshness and eating qualities.

activities and eliminate bacteria in seafood products. High pressure processing applied to shucking of raw oysters eliminates pathogenic bacteria. MAP has expanded markets for fresh pre-packaged fis fille and portion. Both technologies retard or eliminate natural autolytic enzyme activities or reduce growth of spoilage microbiota present in chilled-stored fish The successful application of these techniques depends upon the pre-harvest history of products well before the fis reaches the processing steps.

3.5   Environmental taints

On occasion fis will acquire off-fl vours that are not natural in the fresh to stale continuum but attributed to feeding conditions or natural compounds in the environment. A well-known off-fl vour is the muddy-earthy taint in many freshwater fish especially in North American catfis [24]. The fl vour is mainly caused by two compounds, geosmin and 2-methyl-iso-borneol. Geosmin is produced primarily by blue-green algae, Streptomycetes, and Actinomycetes. Another environmental taint is the iodine-like fl vour found in some marine fis and shrimp. This is caused by the volatile bromophenolic compounds formed by marine algae, sponges, and Bryozoa and are taken up through the diet of fis at certain locations and times of the year. Other taints include oil fl vour/odours due to crude oil spills and spillage from vessels in coastal waterways. The fraction that is soluble in water is responsible for the off-fl vours, where particularly the aromatic compounds are responsible
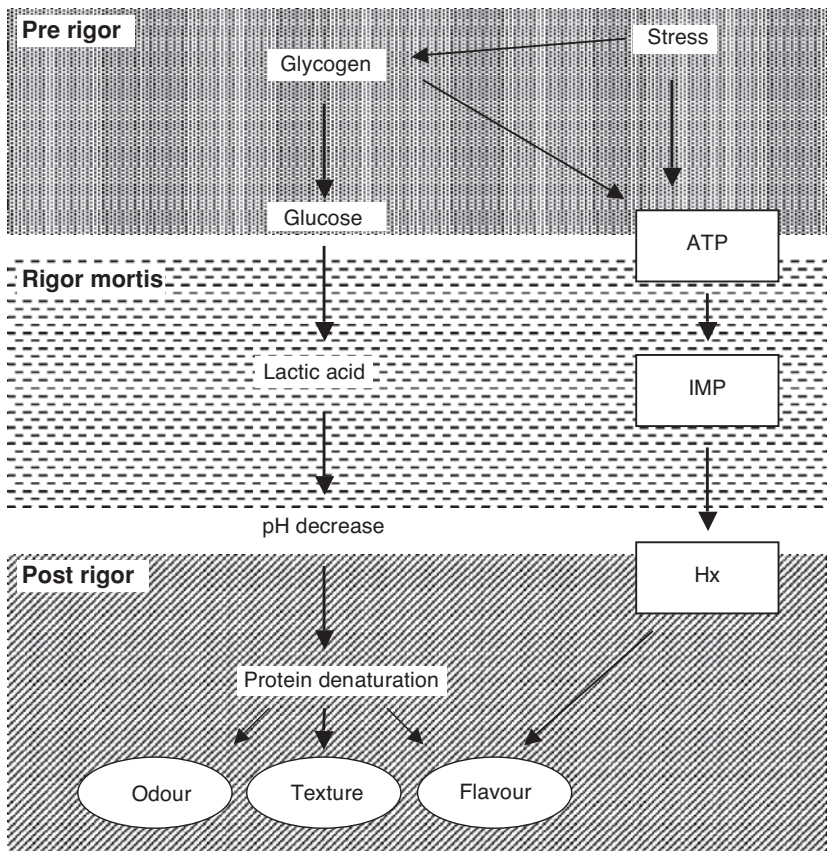
Fig. 3.2   Post-mortem changes in fish muscle due to autolytic activity. ATP, adenosine 5'-triphosphate; IMP, inosine 5'-monophosphate; Hx, hypoxanthine.
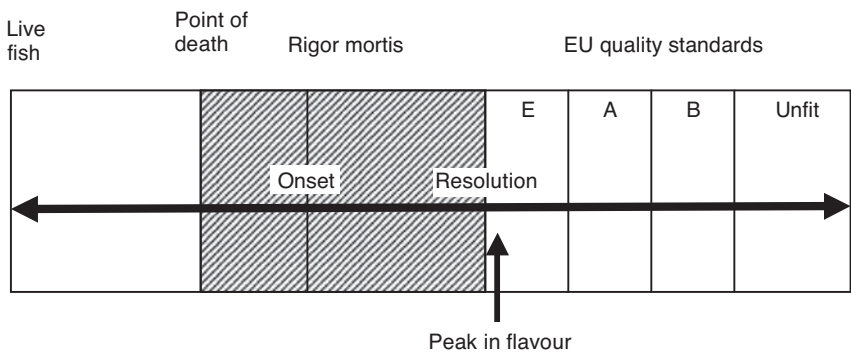
Fig. 3.3   Schematic of the fresh to stale continuum in freshness of chill-stored fish.

[25]. Others include a bitter taste in herring caused by larvae of *Mytilus sp*. [26] and mineral oil or petroleum fl vours in fis caused by the plantonic mollusc, *Spiratell helicina* [1]. The occurrence of off-fl vours due to the presence of environmental taints falls outside the general QIM scheme and are not included in the scoring system. These defects should be noted in quality control but are not associated with the natural deteriorative changes that occur in chill-stored fish

3.6   Extending freshness and shelf-life in fish

Prolonging fis freshness and delaying seafood spoilage are challenges for the seafood industry. Fish "freshness" is estimated by a combination of sensory attributes: appearance, smell, texture, and taste. Sensory evaluation by trained assessors is an accurate way to measure freshness. Quantitative chemical, biological, and instrumental methods techniques are correlated with sensory qualities to evaluate fis freshness and quality attributes. In post-mortem fish intrinsic chemical and physical changes due to endogenous enzyme activity and extrinsic changes due to microbial growth and metabolism cause loss of fis freshness and quality attributes. Improving our understanding of factors affecting post-mortem changes occurring in fis is important for preserving fresh quality and extending shelf-life. Applying these principles in wild harvest and aquaculture practices and in post-harvest handling of fis will help industry supply safe, high-quality, and health promoting fis products.

3.7   Conclusions

Sensory evaluation of fis freshness and eating qualities involves both objective and subjective testing methods. Because different species and fisher products spoil in different patterns, the appropriate use of sensory and non-sensory assessment methods is warranted. The integration in assessment of intrinsic qualities by sensory testing and extrinsic factors may soon lead to prediction of consumer behaviours towards a particular fis product.

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4 Sensometric and chemometric approaches to seafood flavour

Kae Morita and Tetsuo Aishima

4.1 Introduction

In the last decade, consumption of various seafoods has increased rapidly and globally due to the shortage of other animal protein resources and increase in health consciousness. Accordingly, seafoods have gained popularity in many countries, partly because of their high content of polyunsaturated fatty acids (PUFA). However, the most important reason for increase in seafood consumption is their attractiveness and variety of seafood fl vour. Besides *sushi*, a typical raw seafood dish in Japan, people have found attractiveness in cooked fis as well. Generally, fresh raw seafood emits a weak smell but a strong and appetizing aroma is thermally generated through the cooking process. Regardless of whether raw or cooked, aroma notes in individual species of seafood are unique, but some characteristics are commonly shared.

In fl vour research, instrumental analyses supply chemical and physical knowledge but sensory evaluation can offer indispensable information on food quality itself. Since the end of World War II, various sensory techniques have been developed to analyze food quality [1]. According to objectives, sensory analyses can be classifie into two categories, difference tests and descriptive analyses. Simple difference tests, such as the triangle and the duo-trio tests, can show whether overall differences exist between products, but the reason of differentiation is not the issue in these methods. Other types of difference tests, such as the paired comparison test and ranking test, can reveal how an attribute differs between products [1]. However, the so-called quantitative descriptive sensory analysis (QDSA) can supply more abundant and useful information on sample quality than any difference test can. In QDSA, an expert panel consists of well trained panellists qualitatively and quantitatively describing sensory profile that make it possible to compare sample qualities objectively. Since the fully acceptable comparison results of European and American beer fl vours [2], the quantitative descriptive analysis (QDA$^{®}$) and its derivative methods have widely been employed to describe sensory profile of various foods and drinks, including seafood.

Flavour data ordinarily obtained from both instrumental and sensory analyses are highly multidimensional because constituents of food are very complicated in Nature. Multidimensional data cannot be directly observed and is difficul to interpret as it is. "Chemometrics" is the area consisting of versatile mathematical and statistical techniques, such as experimental

design, pattern recognition, and calibration, to conduct chemical experiments efficientl and extract useful information from multidimensional chemical data. Since the late 1960s, multidimensional techniques, such as multiple linear regression analysis (MLR) and linear discriminant analysis (LDA), have been widely applied in fl vour research [3,4]. Food and fl vour research has been recognized as one of the main topics in the chemometrics area [5]. Two volumes edited by the chemometrics group in Belgium and the Netherlands cover full details of the entire chemometric techniques from basics and fundamentals to applications [6]. This chapter highlights sensometric and chemometric approaches to seafood fl vour. Some of the applications conducted by our research group are also mentioned.

4.2 Sensometric approach to seafood flavour

QDSA has been widely applied to research on various food fl vours but only a limited number of papers have reported descriptive sensory data of seafood fl vour. Phleger *et al*. [7] used the fl vour profil method to compare scallop samples using 32 attributes, including 9 aroma attributes. Sensory profile of boiled fl vour of oyster, clam, and shrimp have been illustrated using 29, 22, and 22 attributes, respectively [8]. Using the fl vour profil methods, sensory characteristics of 18 species of snapper and rockfis [9] and 17 species of North Atlantic fis [10] were compared.

Data obtained from the fl vour profil method cannot be analyzed statistically due to the basic principle of methodology, and to overcome this disadvantage QDA was developed [2]. The basic concept of QDSA is similar to that of chromatographic analysis, as schematically illustrated in Fig. 4.1 [11]. In the firs step of QDSA, by using a carefully selected and well trained panel generally composed of 6 to 15 members, all sensory attributes that exist in food samples are identifie through sniffing tasting, and intensive discussions by all panel
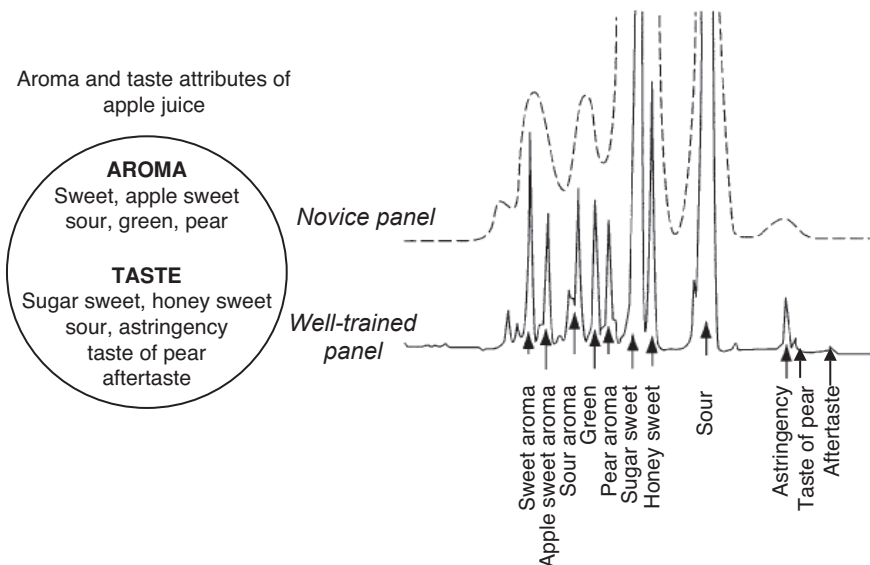
Fig. 4.1 Schematic presentation for basic principle of descriptive sensory analysis exemplified by apple juice flavour. Adapted from Aishima [11]. Copyright 2004, with permission of Elsevier.

members. In the evaluation step, panel members individually quantify strengths of all of the identifie   attributes in samples using a category or line scale [1]. Then scores given for each attribute by panel members are averaged. Morita *et al*. [12] compared boiled fl vour of 10 saltwater fish 3 freshwater fish 2 anadromous fish and a brackish water fis by QDSA using 10 attributes. Flavour profile for boiled squid, prawn, and scallop were successfully described using 10, 10, and 9 attributes, respectively [13–15].

4.3   Chemometric approach to seafood flavour

4.3.1   Experimental designs and optimization

Generation of aromas in cooked seafood is affected by various factors such as species, parts of seafood body, pH, ingredients, and heating conditions. Experimental designs [16] are useful to screen essential factors from numerous possible factor candidates and to optimize cooking conditions. Response surface methodology (RSM) has been applied to optimize the sensory quality of various foods, such as sweetened mango pulp [17], extra virgin olive oil [18], shrimp meat [19], and chocolate peanut spread [20]. In a seafood fl vour study, three-level full-factorial designs (3^2 FFD; [16]) and RSM were employed to investigate the optimum cooking conditions to generate aroma attributes identifie   in boiled squid, prawn, and scallop aroma [13–15].

Response surface is a three- or multidimensional curvature surface covering responses derived from an MLR function composed of the interactive, first- and second-order factors [16]. In the RSM, experiments are conducted at the factor levels define   by factorial, Box-Behnken, or central composite designs. Factor combination for 3^2 FFD for two factors affecting squid fl vour is shown in Fig. 4.2. A second-order RSM model for two factors, x_1

		Factor level		
		−1	0	1
pH	x_1	2	6.5	11
parts	x_2	skin	skin & muscle	muscle
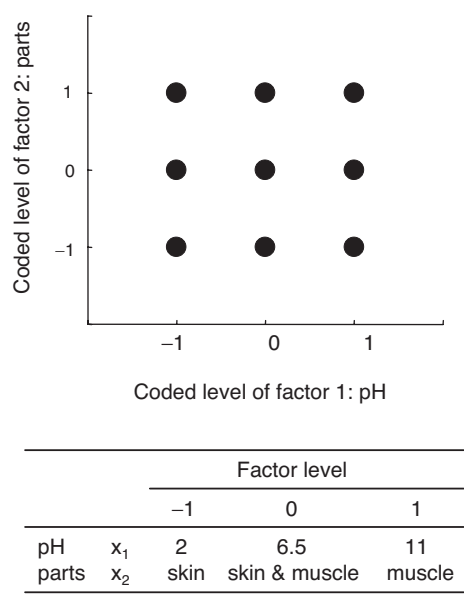
Fig. 4.2   Factor combinations for 3^2 FFD for squid samples.

(a)

(b)

Roasted shrimp
$= 53.48 - 0.066 \times \text{pH} - 0.123 \times \text{part}$
$- 0.975^{***} \times \text{pH}^2 - 0.111 \times \text{part}^2$
$- 0.198 \times \text{pH} \times \text{part} \ (R^2 = 0.927)$

Irritate
$= 13.87 + 0.658^{***} \times \text{pH} + 0.283 \times \text{part}$
$+ 0.523^* \times \text{pH}^2 - 0.172 \times \text{part}^2$
$+ 0.357 \times \text{pH} \times \text{part} \ (R^2 = 0.792)$
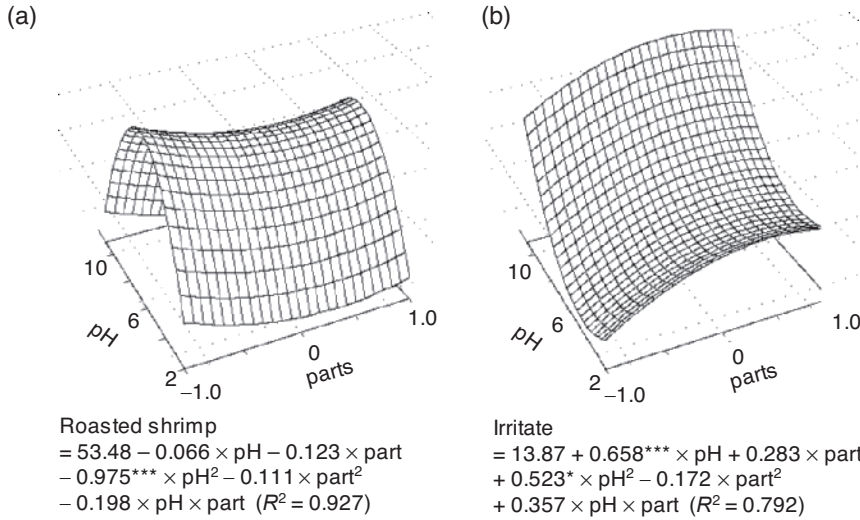
Fig. 4.3 Response surfaces and MLR models for attributes. On the axis for parts, −1, 0, and 1 indicate skin, skin and muscle, and muscle, respectively. In equations, *, **, and *** indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. (a) "Roasted shrimp", (b) "Irritate". Adapted from Morita *et al.* [14], with permission of Blackwell Publishing Ltd.

and x_2, is expressed as Eqn. 4.1:

$$y = b_0 + b_1 x_1 + b_2 x_2 + b_{11} x_1^2 + b_{22} x_2^2 + b_{12} x_1 x_2 \qquad (4.1)$$

where y, x_i, and b_{ij} are the response, factors, and weights or regression coefficients respectively. Weights on factors are calculated by MLR analysis. As shown in Fig. 4.3a, an MLR model for "roasted shrimp" in squid is a ridge shaped response surface due to negative and large second-order effects of pH. On the other hand, RSM for "irritate" are mainly influence by pH alone (Fig. 4.3b). Thus RSM is a useful technique to examine how aroma characteristics in boiled seafood were influence by factors that had been selected through a preliminary experiment using a screening experimental design [13,14].

4.3.2 Pattern recognition

Pattern recognition techniques are divided into two categories; unsupervised and supervised techniques. Unsupervised techniques, including principal component analysis (PCA) and cluster analysis, are used for clustering variables or samples into groups on the basis of their similarity or dissimilarity in the multidimensional space. Supervised techniques, such as LDA, soft independent modelling of class analogy (SIMCA), and partial least squares discriminant (PLSD) analysis, are used for differentiating samples into known groups and then assign unknown samples into known groups using the calculated mathematical model [6]. Among them, PCA is a powerful tool to explore the latent structure hidden in multidimensional data obtained from sensory and/or instrumental analysis of food fl vour. In PCA, an original data matrix is decomposed into a factor loading matrix and a score matrix so as to make the information loss minimal. A so-called "biplot" is an informative plotting method because this plot clearly visualizes mutual relationships between samples and
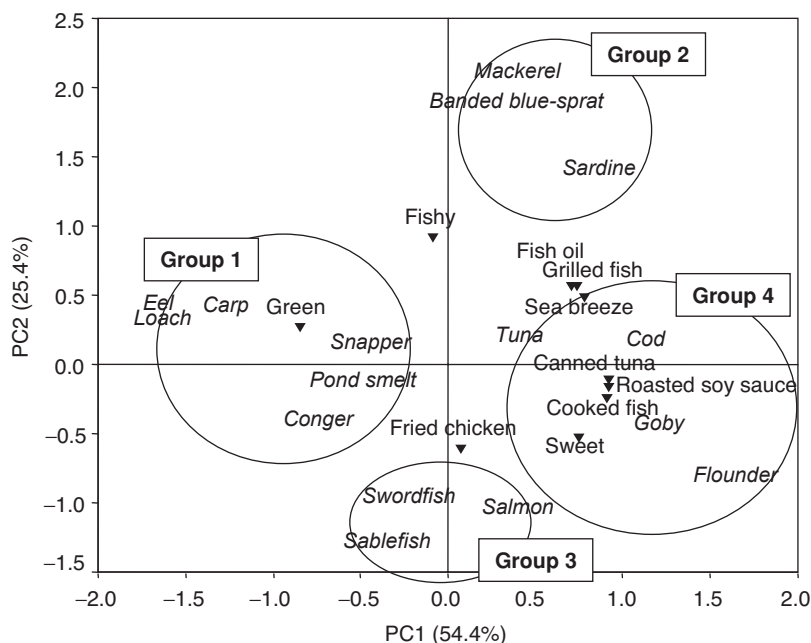
Fig. 4.4   Biplot of PC scores and factor loadings in PCA for broths of 16 fish species. Adapted from Morita *et al.* [12], with permission of Blackwell Publishing Ltd.

variables. This informative biplot is illustrated when a two-dimensional factor loading plot was superimposed onto the corresponding scores plot. Figure 4.4 is a biplot illustrating the mutual relationships between 16 boiled fis and 10 aroma attributes. As shown in this figure information contained in 10 aroma attributes is extracted into two principal components with only 20% loss of information or variance [12]. On the biplot, 16 fis species are classifie into four groups:

- group 1: loach, pondsmelt, carp, eel, snapper, and conger;
- group 2: sardine, banded blue-sprat, and mackerel;
- group 3: swordfish sablefish and salmon; and
- group 4: flounde , cod, tuna, and goby.

Group 1 was found close to "green". Group 2 consisted of migratory coastal fis species located near "fis oil", "grilled fish" "sea breeze", and "fis y". "Fried chicken" were situated adjacent to group 3, and group 4 was found around "cooked fish" "sweet", "canned tuna", and "roasted soy sauce". Although the saltwater and freshwater fis were not separated clearly, all freshwater fis belonging to group 1 were characterized by the "green" note [12].

4.3.3   Multivariate regression analysis

MLR has been the classical and most popular multivariate regression analysis, but MLR essentially suffers from serious limitations and problems due to its algorithm. If independent variables would be highly correlated mutually, that is, multicollinearity, neither reliable nor

logical results could be obtained [21]. Furthermore, MLR cannot be applied to a data set when the number of predictor variables (*m*) exceeds the number of samples (*n*). To overcome these limitations and problems of MLR, the principal component regression (PCR) analysis and then the partial least squares regression (PLSR) analysis were originated in the mid-1980s [22]. Especially from the PLSR analysis, highly predictable models and abundant information on the contribution of each variable to the prediction are obtained. This information can be a great help in investigating relationships latently existing between aroma attributes and numerous volatile components.

In seafood fl vour research, many studies to determine aroma-active compounds have been conducted by employing the aroma extract dilution analysis (AEDA) for boiled trout [23], cod [24], salmon [25], boiled squid [26], cooked crustaceans [27], carp [28], sea bream [29], sardine [30], and tench [31]. However, until now, no single component responsible for overall aroma characteristics in individual seafood has been found. The AEDA is a rather laborious methodology and so PLSR was applied to correlate gas chromatography/mass spectrometry (GC/MS) data to QDSA data in order to fin peaks that influenc seafood aroma [12–15].

Table 4.1 shows peaks selected by PLSR as influentia in each of 10 attributes found in boiled fis aroma. All PLSR models calculated for each attribute consisted of 20 peaks selected statistically from the entire 120 peaks with highly predictable ($R^2 > 0.936$) [12].

4.3.3.1 *Green*

Aldehydes, such as (*E,E*)-2,4-nonadienal and (*E,E*)-2,4-decadienal, were positively related to "green", and these compounds were reported as potent odourants in boiled trout [23] and carp aroma [28].

4.3.3.2 *Grilled fish*

Many sulphur- and/or nitrogen-containing compounds, widely known as thermal degradation products of amino acids, positively relate to "grilled fish" Odour descriptions of 3-ethyl-2,5-dimethylpyrazine and 3-methylthiopropanol were reported as "roasted/nutty/baked potato" and "sweet/soup-like/meat-like", respectively [32].

4.3.3.3 *Fried chicken*

Many sulphur- and/or nitrogen-containing compounds contributed positively to "fried chicken" fl vour. Among them, 2,5-dimethylpyrazine, 2,3,5-trimethylpyrazine, and 3-methylthiopropanal have been reported as potent odorants in trout [23], crayfis waste [33], squid [26], clam [34], and crustaceans [27]. 3-Methyl-1-butanol relating negatively to "fried chicken" character was found in lower-grade canned pink salmon [35].

4.3.3.4 *Cooked fish, sweet, canned tuna, and roasted soy sauce*

Some compounds were commonly related with these four attributes. Among them, seven compounds, including four ketones and a furanone, relate positively to "sweet" and "cooked fish" Pyridine and 2,6-dimethylpyrazine contribute positively to "cooked fish" Seven aldehydes are related negatively to "sweet", "cooked fish" and/or "canned tuna".

Table 4.1 Compounds selected by PLSR as closely relating to each attribute in fish samples. Adapted from Morita *et al*. [12], with permission of Blackwell Publishing Ltd

Peak no	Compound	Positively relating to	Negatively relating to
2	1-Methylpyrrole	Sea[a]	
3	(*E*)-3-Penten-2-one	Soy sauce[b], Sweet, Grilled[c], Tuna[d], Sea, Cooke[e]	Green
6	3-Penten-2-ol	Fried[f]	Fishy
7	Pyridine	Soy sauce, Tuna, Cooked	
9	Unknown		Fish oil
10	3-Methyl-1-butanol		Fried
13	Unknown		Tuna, Cooked
15	*N*,*N*-Dimethylaminoacetonitrile	Fried	Green
19	1-Methylthiopropane	Green, Fishy, Sea	Sweet
20	Unknown	Fish oil	
21	Methylpyrazine	Soy sauce	
23	1-Hydroxy-2-propanone	Fish oil, Soy sauce, Sweet, Tuna, Sea, Cooked	Fishy
25	3-Methyl-2-pentanol		Soy sauce, Grilled, Sea
29	2,5-Dimethylpyrazine	Fried	
31	N,N-Dimethylformamide	Fried	
32	2,6-Dimethylpyrazine	Tuna, Cooked	
34	2,3-Dimethylpyrazine	Fried	Fishy
36	Alkylalcohol	Fish oil, Sweet, Grilled, Tuna, Cooked	Green
37	1-Hydroxy-2-butanone	Soy sauce, Sweet, Tuna, Cooked	Green, Fishy
42	2,3,5-Trimethylpyrazine	Fried	Fishy
46	2,5-Dimethyl-3-ethyl-pyrazine		Sea
47	3-Methylthiopropanal	Fish oil, Fried	
51	2,3-Dimethyl-5-ethylpyrazine	Grilled, Fried	
56	2-Ethyl-2-hexanol		Tuna
58	Benzaldehyde		Fish oil, Grilled, Fishy
59	2-Methylthioethanol	Soy sauce	
60	Unknown	Fish oil, Fishy	
62	5-Methylfurfural	Green	Soy sauce, Tuna, Sea, Cooked
68	2-Undecanone	Grilled	Green
69	Undecanal	Green, Fishy	Soy sauce, Sweet, Tuna, Cooked
70	(*Z*)-2-Decenal	Green	Sweet
71	2-(2-Ethoxyethoxy)-ethanol		Green, Fried
73	Butanoic acid	Fish oil, Soy sauce, Grilled, Sea	
75	(*E*)-2-Decenal		Sweet
76	2-Acetylthiazole		Tuna
78	Furfuryl alcohol	Fishy	
79	1-Nonanol		Fishy
82	(*E*,*E*)-2,4-Nonadienal	Green	Soy sauce, Cooked
83	4-Ethylbenzaldehyde	Fish oil, Grilled, Sea	
84	3-Methylthiopropanol	Grilled, Sea	

(*Continued*)

Table 4.1 (*Continued*)

Peak no	Compound	Positively relating to	Negatively relating to
86	3,4-Dihydropyran	Fish oil, Soy sauce, Sweet	
87	(*E*)-2-Dodecenal	Green	Sweet, Cooked
88	5-Ethyl-2(5*H*)-furanone	Tuna	
89	(*E,Z*)-2,4-Decadienal	Green	Cooked
90	Unknown	Fried	
91	2-(2-Butoxyethoxy)-ethanol	Fish oil, Grilled, Sea	
92	Dihydro-5-propyl-2(3*H*)-furanone	Sweet, Fried	
93	(*E,E*)-2,4-Decadienal	Green	Soy sauce, Sweet, Tuna, Cooked
94	3-Thienylmethanol		Grilled
97	Geranyl acetone		Fish oil, Soy sauce, Grilled, Fried, Cooked
99	2,4,4-Trimethyl-3-hydroxypent-1-yl isobutyrate		Cooked
100	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	Fish oil, Soy sauce, Sweet, Grilled, Tuna, Sea, Cooked	Green
101	2,4,4-Trimethyl-5-hydroxypent-3-yl isobutyrate	Fish oil, Sweet, Sea	
103	2-Phenylethanol		Sea
104	5-Butyldihydro-2(3*H*)-furanone	Fish oil, Soy sauce, Sweet, Tuna, Fried, Cooked	Green
105	2,4-Undecadienal	Fishy	Sweet, Tuna, Fried
106	2-Phenyl-2-butenal	Soy sauce, Grilled, Fried	Green
107	Unknown	Fish oil, Grilled, Fishy	
108	Alkylpyridine	Fishy	
110	2-Acetylpyrrole		Green
111	Dodecanol	Fishy	
112	Alkylpyridine	Fishy	
113	Phenol	Fish oil, Soy sauce, Sweet, Grilled, Fishy, Tuna, Sea	Green
114	2-(1-methylpropyl)-phenol	Fish oil, Grilled, Fishy	
115	2-(1-Methylpropyl)-cyclopentanone	Grilled, Fishy, Sea	
116	2-Pentadecanone		Sea
117	Dihydro-5-pentyl-2(3*H*)-furanone	Fried	Fish oil, Soy sauce, Tuna, Sea, Cooked
118	Unknown	Green, Fishy	Fish oil, Soy sauce, Sweet, Grilled, Tuna, Sea, Fried, Cooked
120	Octnoic acid	Sweet, Tuna, Sea, Cooked	

[a]Sea breeze, [b]roasted soy sauce, [c]grilled fish, [d]canned tuna, [e]cooked fish, and [f]fried chicken.

4.3.4 Compound-sensory mapping

A derivative of PLSR analysis called PLSR2 can directly correlate two multidimensional data matrices. PLSR2 seems to be useful in exploring the mutual relationships between QDSA data composed of 10 attributes and GC/MS data.

Two data sets, sensory scores composed of 10 attributes and influentia peaks, listed in Table 4.1, were analyzed by PLSR2 to obtain a compound-sensory map that illustrates overall
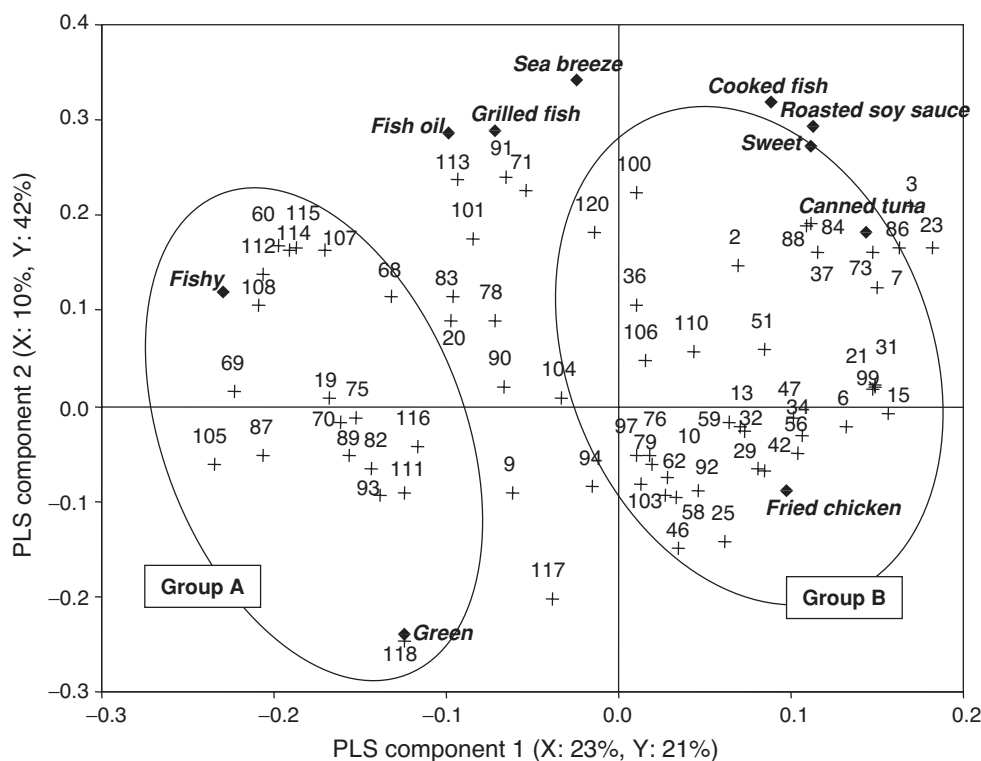
Fig. 4.5 Compound-sensory mapping for fish samples.

mutual relationships between them (Fig. 4.5). Group A consisted of aldehydes and is found on the left-hand side, located close to "fis y" and "green". However, on the right-hand side, pyrazines generated thermally belonging to group B were found near to attributes describing cooked fis aroma. This suggests that the aroma of boiled fis consists of various smells in raw materials and aromas generated thermally, and differences in their profile make boiled fis aroma unique to each species.

4.4 Conclusions

Chemometric techniques, which have been widely applied to extracting information from complicated chemical data, showed their effectiveness to efficientl conduct research on seafood fl vour. QDSA profile clearly demonstrated differences and similarities in seafood fl vour. QDSA provides quantitative sensory data similar to that obtained from instrumental analysis. It suggested that QDSA and instrumental data could be handled similarly and were connected to each other. Both chemical composition and sensory properties of any cooked aroma will be complicated in nature. Therefore, designed experiments coupled with RSM make it possible to efficientl explore factors essential for generating unique aromas in cooked seafood. PLSR models composed of peaks statistically selected as influentia for each attribute are highly predictable but a PLSR2 biplot clearly illustrated overall relationships

among seafood species, sensory attributes, and volatile compounds. Although currently not so popularly utilized, fully incorporating versatile chemometric techniques into research strategy for seafood fl vour will be a great help in designing well organized experiments and to extract useful information efficientl from complicated data matrices.

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5 Instrumental analysis of seafood flavour

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5.1 Introduction

The complex fl vour of seafood is composed of equally important nonvolatile taste- and aroma-active components. Early investigations on seafood fl vour focused mainly on the taste-active components, which are generally non-volatile and low-molecular-weight extractive components. These may be divided into two broad groups: nitrogenous compounds including amino acids, low-molecular-weight peptides, nucleotides, and organic bases; and non-nitrogenous compounds including organic acids, sugars, and inorganic constituents such as mineral salts [1,2]. Study of the taste active constituents has attracted considerable attention and their importance to seafood fl vour has been thoroughly reviewed [3–6]. The firs study of this type was conducted during the early 1900s [7]. Since that time, most investigations in this area have involved the quantitative analysis of extractive components (mainly nucleotides and free amino acids) by wet-chemical and/or liquid chromatographic methods, including ion exchange chromatography and high-performance liquid chromatography [6,8–14].

Volatile (aroma) constituents are key to fl vour perception. Without aromas, it is very difficul to identify the fl vour of specifi food products including seafood [15]. Seafood aromas can be formed via several mechanisms, which may be subdivided into four categories according to a precursor-mechanism relationship:

1) enzyme-mediated conversion of lipids to aromas;
2) autoxidative degradation of free fatty acids leading to the formation of volatile carbonyls, acids, and alcohols;
3) enzymatic conversion of sulphur- and nitrogen-containing precursors to volatiles including dimethyl sulphide; and
4) thermal decomposition of precursors upon processing or cooking [16].

The aroma components may contribute to the development of pleasant (characteristic fl vour) or off-fl vour characteristics of seafood. The characteristic fl vour of seafood has been described as green, melon-like, and iodine-like, while off-fl vours include musty, fis y, woody, rancid, and petroleum notes [4,17]. Research on the volatile constituents of seafood

has markedly increased since the introduction of gas chromatography (GC) coupled with GC-mass spectrometry (GC-MS) in the mid-1960s. Aroma is one of the most important determinants of seafood quality and can profoundly affect consumer acceptability [1,17,18]. As mentioned above, the fl vour of seafood is comprised of both volatile aroma-active and non-volatile taste-active components. The identificatio of volatile constituents of seafood, therefore, is a key firs step to the full understanding of seafood fl vour. This chapter focuses on procedures for isolation and extraction of volatile fl vour components and describes recent advances in analytical methodology for characterization of seafood fl vour.

5.2   Isolation of volatile flavour compounds

Analysis of volatile fl vour components in food is complicated due to the presence of extremely low levels of volatile solutes in highly complex nonvolatile matrices. Isolation or sampling of volatiles should be conducted by taking advantage of their volatility or nonpolar nature prior to GC analysis [18]. There are numerous methods for isolation of volatiles from a food matrix. Methods most often employed in the analysis of volatile fl vour components of seafood are summarized in Table 5.1 and discussed below.

5.2.1   Headspace sampling

Headspace sampling techniques take advantage of the volatility of aroma compounds, and involve several categories including static headspace, dynamic headspace (purge-and-trap), solid phase microextraction (SPME), solid phase aroma concentration extraction, in-tube sorptive extraction, and headspace sorptive extraction. In each case, however, the same fundamental principle is employed; only volatile compounds are collected from the atmosphere adjacent to the sample, leaving the actual sample material behind.

5.2.1.1   *Static headspace sampling*

Static headspace sampling (SHS) is the simplest among the headspace techniques. In SHS, the sample is placed in an airtight vessel (vial) and the volatile components are allowed to come to equilibrium between the sample matrix and the surrounding headspace. The equilibrium is affected by the temperature of vessel, sample size, and equilibration time, etc. [19]. Following this, the headspace vapour (0.1–2.0 mL) is injected into a GC using a gastight syringe or by direct transfer to the injection port using a headspace sampler (sampling loop). SHS is covered in greater depth elsewhere [23]. Advantages of SHS include simple sample preparation, low risk of artifacts, and elimination of reagent or organic solvent. The technique allows for the analysis of highly volatile low molecular weight aroma compounds in seafoods, such as acetaldehyde, methanthiol, trimethylamine, dimethyl sulphide, or 2-methylpropanal [36].

SHS has been used to identify volatile compounds in fis oil [37], salmon [38,39], white herring [40], and other fis species [41]. However, SHS is mainly used in the fiel of quality control or grade classificatio of seafood products by the analysis of certain target volatile compounds, such as trimethylamine.

Table 5.1 Methods used for the isolation of the volatile flavour constituents of seafood products

Isolation method	Principles of the technique	Advantages	Disadvantages
Headspace sampling			
Static headspace sampling (SHS)	Volatile analytes contained in the headspace gas phase under equilibrium are sampled by a gastight syringe or other means and transferred to the GC column for analysis [20].	Direct and non-destructive analysis of the volatile analytes [21]. Easy elimination of interferences from the complex sample matrix. Ability to analyze low molecular weight volatiles without the presence of a solvent peak [22]. Relative low cost per analysis, easy automation, and simple and fast isolation of the volatiles [21,22].	Withdrawing and transferring only small portion of the headspace (1–2 mL) results in poor sensitivity for trace level volatile analytes [22,23]. Not suitable for isolating of volatiles with high boiling points [22].
Dynamic headspace sampling (DHS)	Carrier gas containing the volatile analytes above the sample (headspace) is constantly swept through a trap, and the volatiles are retained on the trap, which results in the concentration of the analytes [22]. DHS was developed to overcome the sample size limitation imposed by SHS [21].	Increased total volume of headspace (100 mL–1 L), which may result in higher recovery of the analytes and provides greater sensitivity than SHS [22].	Requires more complex and expensive instruments, such as additional thermal desorption and cryofocusing systems. Lengthy analysis time due to more analytical steps, including sample purging, trap drying, trap transfer, and thermal desorption of trap [22].
Solid phase microextraction (SPME)	The analytes in the vapour phase are absorbed/adsorbed by a small volume of an extracting phase (<1 µL), which consists of thin polymeric films coated onto fused silica fibres protected in a needle of a syringe-like device [24].	Rapid, simple and easy to automate for the extraction of both polar and non-polar volatiles. Higher sensitivity towards volatile organic compounds compared with SHS or DHS [25]. Advantage of direct thermal desorption into the GC injection port.	Small volume of the extraction film allows for only volatiles having high partition coefficient to be extracted with high efficiency. SPME fibres are relatively expensive, and the polymer coating is fragile and easily broken [26]. Limited lifetime of the fibres (up to 100 analyses). Sample carryover is sometimes difficult to eliminate [26], and some extraneous peaks are formed due to partial decomposition of fibre coating [27].

Method	Principle	Advantages	Disadvantages
Sorptive extraction	Similar principle to that of SPME except using higher mass of polymeric film (25–300 µL) [28]. Overcomes the limited concentration capability of SPME [28].	Higher recoveries and higher sample capacity, which leads to lower detection limits (capability of isolation of trace volatiles) and better repeatability than other headspace techniques [29].	Limited number polymeric extraction films available, which restricts the method to the isolation of mainly non-polar volatile compounds (i.e. only non-polar compounds are extracted with the available PDMS coating) [30].
Solvent extraction and distillation extraction			
Direct solvent extraction (DSE)	The analytes are isolated from food matrix by extraction with organic solvent taking advantage of the difference in polarity.	Simple, no need for complex equipment and large selectivity and flexibility [31].	Requiring additional clean-up step in order to remove non-volatile residues. Emulsion formation, which may lead to loss of analytes, and requires complicated and time-consuming alternative steps to prevent or minimize [31].
Steam distillation extraction (SDE)	SDE takes advantage of volatility of the analytes and non-volatility of other major food constituents [32].	High recovery of steam-distillable volatiles [33]. Simplicity of operation, reproducibility and applicable to broad range of samples [31].	Possible decomposition of volatiles or production of artifacts due to presence of water and high extraction temperature [34]. Poor recovery for polar and water-soluble analytes [35].
High vacuum distillation extraction	The pressure above the acqueous sample mixture to be distillated is reduced to less than its vapour pressure causing evaporation of volatile analytes including solvent or water.	High yield of volatiles including polar volatiles, and recovery of authentic flavor extracts [35].	Chance for loss of the highly volatile trace analytes.

5.2.1.2 *Dynamic headspace sampling*

Dynamic headspace sampling (DHS) or purge-and-trap analysis involves the constant stripping of the volatile analytes in the atmosphere surrounding a sample by use of an inert carrier gas such as nitrogen. The volatiles contained in the carrier gas are then enriched by trapping onto adsorbent materials (generally porous polymers) or by cryogenic focusing. This technique greatly improves the efficien y of headspace sampling. In general, the term "purge-and-trap" is used when referring to liquid samples analyzed by bubbling the carrier gas through the liquid, while DHS is used when the sample is a solid [22]. Tenax® (poly-2,6-diphenyl-*p*-phenyl oxide) is the most widely used adsorbent material for DHS. However, the adsorbent material can be chosen according to the specificit of the target volatile analytes. The volatile analytes are desorbed (released) by heating the trap (thermal desorption), and the released volatiles are sent to the analytical GC column for analysis. DHS has many of the same advantages as SHS. Furthermore, volatiles isolated by DHS may more closely resemble the actual aroma composition that is perceived during smelling. A major disadvantage of DHS is that it is not efficien towards components of low volatility [19]. DHS is one of the most popular isolating techniques for seafood fl vour analysis. DHS has been used by several researchers for the isolation of volatiles from various kinds of seafood, such as sea bream [42], herring [43], cooked lobster tail meat [44,45], boiled crayfis [46] and its waste [47], emerald shiner [48], and pickled fis [49].

5.2.1.3 *Solid phase microextraction*

Solid phase microextraction (SPME) is a relatively new technique for the rapid, solventless extraction of volatile compounds based on their partitioning between the sample or sample headspace and a polymer-coated fibre The fibr is attached to a stainless steel plunger, sheathed by a protective needle, which is essentially a modifie syringe to enable thermal desorption of the analytes into a GC injection port. The selectivity of volatile extraction from the headspace depends on the choice of the fibre and two factors, such as polarity and volatility and molecular weight of target analytes, need to be considered [30]. SPME is an equilibrium technique and therefore the volatile profil one obtains is strongly dependent upon sample composition, and careful control of all sampling parameters is required [15]. Comprehensive reviews of SPME have been published elsewhere [50–52]. Recently, solid phase aroma concentration extraction (SPACE™) was introduced as a modificatio of SPME, with the aim of increasing the area of the adsorbent so as to improve sensitivity (over 30 times more than SPME) [30,53]. SPACE™ consists of a stainless steel rod coated with a mixture of adsorbents, mainly graphite carbon [28]. Use of SPME for the analysis of seafood fl vour is limited [54], with most applications related to the monitoring of quality control factors such as freshness and spoilage indicators rather than analysis of total volatiles [55–60].

5.2.1.4 *Sorptive extraction*

Among several sorptive extraction methodologies, in-tube sorptive and headspace sorptive extractions (stir bar sorptive extraction) has recently been employed in the fiel of food analysis [61,62]. Both extraction techniques were developed to overcome the relatively limited concentration capability of SPME [28].

In-tube sorptive extraction techniques include solid phase dynamic extraction (SPDE), which is also known as "the magic needle" [63]. SPDE employs a thick fil (50 µm) of

polymer, which is coated onto the inside wall of the stainless steel needle of a gastight syringe, in order to increase sensitivity. The analytes are accumulated in the polymer coating by pulling in and pushing out a fi ed volume of headspace to be sampled, through the gastight syringe for an appropriate number of times within a fi ed time. The trapped analytes are then thermally desorbed into the GC injector [28].

In headspace sorptive extraction or stir bar sorptive extraction (commercialized by Gerstel (Mülheim an der Ruhr, Germany, under the name *Twister*), the headspace analytes are statically accumulated by suspending a polydimethylsiloxane (25–250 µL) coated glass magnetic stir bar in the vapour phase. After sampling, the stir bar is placed in a glass tube and transferred to a thermo-desorption system where the analytes are thermally recovered and analyzed by GC or GC-MS [28]. Although these techniques have not been used for the analysis of seafood fl vour, they have good potential for this application, especially if the analysis is focused on the identificatio of trace level highly volatile or semi-volatile low molecular weight of components.

5.2.2 Solvent extraction and distillation extractions

5.2.2.1 *Direct solvent extraction*

One of the simplest and most efficien techniques for aroma isolation is direct solvent extraction (DSE). DSE takes advantage of the difference in polarity between aroma compounds and food matrix; most volatile aroma compounds are considerably less polar than aqueous food matrix material. Solvent extraction can be as simple as putting a food sample into a vessel such as a separatory funnel, adding a solvent (diethyl ether or methylene chloride are good general purpose solvents), and shaking. The solvent phase is collected, dried with anhydrous salt (e.g. anhydrous sodium sulphate), and then concentrated (using distillation or nitrogen gas purging) prior to GC analysis. Another approach is to use liquid-liquid continuous extractors when relatively large amounts of aqueous samples are available. In the case of solvent extracts prepared from seafood, an additional clean-up step is often required in order to separate nonvolatile residues (e.g. lipid) from the volatile material. This can be accomplished by steam distillation, high vacuum distillation, or DHS. Milo and Grosch [64] performed direct solvent extraction followed by high vacuum distillation for the isolation of volatiles from boiled trout, salmon, and cod. An alternative approach is to isolate the volatile components from the sample by distillation, followed by solvent extraction of the aqueous distillate [15,19].

5.2.2.2 *Steam distillation extraction*

The most common steam distillation method employs simultaneous distillation-solvent extraction (SDE), which is often called the Likens-Nickerson method. In SDE, volatiles are steam-distilled from the sample (an aqueous solution or slurry of a solid material in water) by heating a sample flask and simultaneously the solvent is distilled from another separate flas by mild heating. Vapours condense together on a cold finge where the extraction process occurs between both liquid film on the condenser surface. Water and solvent (containing volatiles) are collected and decanted in the separator, and are finall returned to their respective flask [65]. SDE is often operated under reduced pressure in order to minimize the formation of thermally-induced artifacts. The aroma isolate prepared by SDE contains nearly all the volatiles in a sample, but their proportions may only poorly represent the true volatile

profil of the sample. Despite this, the method is still popular due to its ability to recover volatiles with medium to high boiling points [15]. SDE (atmospheric or reduced-pressure operation) has been widely used for seafood fl vour analysis [45,47,66–73].

5.2.2.3   *High vacuum distillation extraction*

High vacuum distillation, which is one of the early classical techniques, has been applied to isolate low level (ppb to ppt) volatile components of food products containing high fat content. Volatile analytes are distilled from a sample for several hours under high vacuum ($\sim 10^{-5}$ Torr) and mild heat ($<60°C$) conditions, with subsequent condensation of volatiles in a series of cold traps. The volatiles compounds are later recovered from the condensed phase by solvent extraction. Although losses may also occur during extraction and concentration of solvent extract, this technique enables the isolation of a broad range of mid- to high-boiling trace-level fl vour compounds at sufficien quantities for analysis [30]. High vacuum distillation has been used for the determination of aroma-active compounds in cooked tail meat of lobster [44]. Engel *et al.* [74] developed a new technique called solvent-assisted fl vour evaporation (SAFE), which allows for faster and more efficien isolation compared with classic high vacuum distillation methods. The future prospects are excellent for the widespread use of SAFE in seafood fl vour analysis.

5.3   Instrumental analysis of volatile flavour compounds

Tandem GC-MS has been the technique of choice for the analysis of volatile food fl vour. GC is ideally suited to deal with solutes in the vapour phase, such as volatile fl vour components [19]. Mass spectrometry is one of the most powerful techniques for identificatio of unknown compounds. Most research conducted on seafood fl vour in the last few years has depended on GC-MS as the main analytical tool. The technique is so standard and routine in fl vour studies of seafood that there is no need to describe it any further here [19].

Standard GC-MS used in fl vour analysis is considered as fused silica, capillary column GC with bonded phase, providing high resolution, combined with fast scanning, high-sensitive MS operating in the electron impact ionization mode [19]. Despite the pre-eminence of standard GC-MS in fl vour research, there are other approaches, which can provide valuable additional and/or complementary information to GC-MS. These approaches are summarized in Table 5.2 and discussed below.

5.3.1   **Gas chromatography**

5.3.1.1   *Gas chromatography-olfactometry (sensory-directed analytical techniques)*

A high resolution GC column coupled with a standard GC detector is capable of separating and detecting hundreds of volatile compounds in a single run. However, it is likely that many of these components have little or no impact on the actual aroma of the food. The aroma-active components in the volatile isolate can be determined by combining GC with olfactometry (GCO). In GCO, the analytes are firs separated by GC and then delivered to an oflactomete (sniffin port) where they are mixed with humidifie air. Human "sniffers" continuously breathe (nasally) the air emitted from the olfactometer, and record the perceived odour descriptions and intensities of the detected odorants. There are several excellent reviews

Table 5.2 Instrumental methods used for the analysis of the volatile flavour constituents of seafood products

Analytical method	Principles of the technique	Advantages	Disadvantages
Gas chromatography (GC)			
Gas chromatography–olfactometry (GCO)	Human panelists are used as GC detectors (use of human subjects for sniffing of GC effluents) [15,75].	Identification of potent odour-active component.	Infeasibility of continuous analysis due to olfactory fatigue. Time consuming due to the high number of assays (dilutions) required. Need for well-trained analysts.
Multidimensional gas chromatography	Two GC columns of different selectivity are linked in series, and the analytes are transferred from the first column (precolumn) to the second column (analysis column) to improve the separation power [30].	Greater reliability in identification of minor compounds in complex samples due to increased peak capacity and separation power [76].	More complex and expensive instrument compared to conventional GC [76]. Elaborate methods development, requiring optimization of many separation parameters [76,77].
Mass spectrometry (MS)			
High resolution mass spectrometry (HR-MS)	HR-MS having a combination of electrostatic (velocity selector) and magnetic (momentum selector) sectors focuses ions according to both direction and velocity while dispersing according to mass-to-charge ratio [78].	Capable of accurate mass measurement, which allows for the determination of elemental composition (molecular formulas) and identification of new compound [15,78].	Need for expensive instruments and a specialist for the operation.
Selected ion monitoring (SIM) mass spectrometry	Only the intensities of selected ions are monitored rather than entire mass spectrum.	Improvement of sensitivity for target compounds.	Retention time (RT)-based technique, which can occasionally cause misidentification of the target analytes due to change of the peak RT during analysis.

(Continued)

Table 5.2 (Continued)

Analytical method	Principles of the technique	Advantages	Disadvantages
Chemical ionization mass spectrometry (CI-MS)	The analytes are ionized by ion-molecule reactions with mostly positive charged reagent gas ions [79].	Not much fragmentation of analytes, which is useful to confirm relative molecular weight [80].	Limited amount of structural information – additional analytical technique or use in combination with EI is required in order to overcome this limitation [81].
Negative ion chemical ionization mass spectrometry	The principle of this technique is very similar to that of positive ion CI-MS only, except for using negatively charged reagent gas ions (such as OH^-).	Higher softness and sensitivity than positive ion CI-MS in many respects [21].	Limited amount of structural information – additional analytical technique or use in combination with EI is required in order to overcome this limitation [81].
Time-of-flight (TOF) mass spectrometry	Created ions from ion source are accelerated by an electric field and allowed to drift through an evaluated field-free region (flight tube) where they separated into groups (isomass packets) according to their mass-to-charge ratio, and the flight time of ions required to reach the detector through flight path is measured and used to calculated mass [82].	Measurment of all of the ions across the m/z range simultaneously, which results in high sensitivity, mass resolution and mass accuracy [82,83].	Blinding effect on multiple concurrent events in case two or more ions arrive at the array detector at the same instant, resulting electrical pulse to be recorded as if only one ion had arrived – adjustment required to correct this effect [80].
Electronic nose (e-nose)	The e-nose functions by analysis of the responses of a sensor array to a complete aroma mixture, which means there is no separation of aroma components [15].	Rapid analysis of flavours without separation step, which is attractive for quality control in the food industry [15,84]. Continuous analysis with no sensory fatigue like with human subjects.	Does not provide any specific detailed chemical information that is possible with GC-MS methods [15,84]. Response of the sensors toward non-target volatiles (i.e. water vapour or CO_2) may alter sensor response patterns [15]. Need for several sensors (3–15) for the analysis and deterioration of the sensors with time [15].

dedicated to GCO [75,85]. Some common methods based on GCO include aroma extract dilution analysis (AEDA) [86], Charm [87], and Osme [88]. These methods mainly differ in how GCO data are recorded and analyzed.

Osme (time-intensity measurement) measures the perceived odour intensity of a compound in the GC effluent The subject rates the aroma intensity by using a computerized 16-point scale time-intensity device and indicates the corresponding aroma characteristics. This technique provides an FID-style aromagram called an osmegram [75]. AEDA and CharmAnalysis (dilution techniques) both rely on GCO of a serial dilution series of an aroma extract. In AEDA, each odour-active compound is assigned a fl vour dilution (FD) factor, which is based on the highest extract dilution at which the odorant was last detected by GCO. FD factors are proportional to the odour unit values (compound concentration/odour-detection threshold). CharmAnalysis differs from AEDA in that the duration of the perceived odour is taken into consideration in the calculation of odour unit values. AEDA has been used to determine potent odorants in hake [89], boiled carp fille [90], cooked turbot [91], skipjack tuna sauce [92], cooked spiny lobster tail meat [45], and boiled cod [93]. The use of CharmAnalysis [18] and/or Osme [94] for the evaluation of seafood fl vour is limited. Other miscellaneous GCO techniques have also been used in the study of seafood fl vour [40,58].

5.3.1.2 *Multidimensional gas chromatography*

With samples as complex as those encountered in a typical fl vour analysis, even with the best high-resolution GC column components sometimes co-elute during GC-MS analysis, producing mixed mass spectra that are difficul to interpret [19]. Multidimensional GC (MDGC), which utilizes two different GC columns (having different selectivities) in series, termed a pre-column and an analytical column, can often overcome this problem [19]. A thorough discussion of MDGC can be found elsewhere [95]. Comprehensive two-dimensional GC, a type of MDGC, was recently developed, which allows greater separation efficien y than traditional MDGC [30]. MDGC has been used in the identificatio of specifi environmental pollutant (PCB and dioxin) in seafood (Baltic herring) and seafood products (fis oil) rather than in the study of seafood fl vour [96,97].

5.3.2 **Mass spectrometry**

Electron impact mass spectrometry (EI-MS) is the most common mass spectral technique used in fl vour analysis, but alternative forms of MS may be employed following other mass spectral techniques for certain specifi problems.

5.3.2.1 *High resolution mass spectrometry*

Mass spectrometers may be classifie as low-resolution (LR) or high-resolution (HR) instruments. The LR instruments provide mass measurements to the closet whole unit mass. Since many combinations of atoms may give the same unit mass, LR-MS may provide the molecular weight of a compound but does not provide elemental composition. HR instruments provide sufficientl accurate mass measurements to permit determination of elemental composition [15]. HR-MS has not yet been widely exploited in seafood fl vour analysis – currently focused on the analysis of environmental pollutants in seafood [98]. However, with the continuous improvements in the performance of commercial magnetic sector and

time-of-fligh mass spectrometers, especially with regard to sensitivity at high resolution, this method will become more readily available in future [19].

5.3.2.2   *Selected ion monitoring mass spectrometry*

In the selected ion monitoring (SIM) mode, the MS continuously measures only selected ions representative of a specifi compound, or group of compounds at very short time intervals throughout a GC run. The technique is extremely useful in enabling a very high sensitivity assay for the known component or types of components in question, but it does not contribute to the identificatio of unknown compounds, since full spectra are not recorded. For instance, geosmin ((E)-1,10-dimethyl-(E)-9-decalol), which is an environmental-related off-fl vour in seafood products (described as earthy-musty) [99], can be detected at trace levels by selecting m/z 97, 112, and 125 with SIM mode [100].

An alternative approach is "mass chromatography", which is useful for deconvoluting co-eluted GC peaks [101]. The difference is that complete mass spectra have been recorded throughout the GC-MS run, rather than selected ions as in SIM. The data analysis system can then be instructed to select appropriate specifi ions from the full recorded spectra of the peak, with the objective of artificiall resolving and recognizing the two (or more) components of the peak [19].

5.3.2.3   *Chemical ionization mass spectrometry*

In conventional EI-MS, sometimes no molecular ion peak is obtained in the mass spectrum of a compound. This may be due to the instability of the molecular ion under the excessive energy imparted by electron impact (an energy of 70 eV is usually employed in EI-MS). If some target compounds in a sample are susceptible to the EI, a softer ionization technique should be employed. Chemical ionization (CI) is the most common alternative, softer ionization approach in GC-MS. In CI-MS, a reagent gas, such as methane, isobutene, or ammonia, is introduced into the mass spectrometer source to be ionized by broadly conventional EI. A range of positive ions, such as $C_2H_5^+$ from methane, is produced. Sample molecules are then ionized by ion-molecule reactions with reagent gas species. The result is that so-called pseudo-molecular ions are produced, such as $(M+H)^+$, by proton transfer. Typically energy of only 5 eV is imparted to sample molecules, so usually very little fragmentation is observed under these conditions. The value of CI-MS in fl vour analysis is to complement and supplement the data provided by EI-MS [19]. CI-MS is commonly used in stable isotope dilution analysis and has been applied to the analysis of important seafood aroma compounds [36,64,102].

5.3.2.4   *Negative chemical ionization mass spectrometry*

In addition to positive ion CI-MS, it is possible to perform negative ion CI-MS, in which negatively charged reagent gas ions, such as OH^-, undergo similar ion molecule interactions with sample molecules, but with the result that negatively charged pseudo-molecular ions are obtained, such as $(M-H)^-$, which is produced by proton abstraction. In many respects, negative ion CI-MS can be superior to positive ion CI-MS, both in terms of sensitivity and degree of "softness". Negative ion CI-MS has not been widely used in fl vour analysis except in the case of target analysis, such as in stable isotope dilution analysis [103].

5.3.2.5 *Time-of-flight mass spectrometry*

The time-of-fligh (TOF) mass spectrometer uniquely offers the ability to take a large number of spectra across a GC peak. This is because TOF instruments employ a detector array for full range mass detection, which means TOF-MS does not scan but rather measures all of the ions across the m/z range simultaneously with a much faster spectra generation rate (50–500 spectra/sec) than other types of spectrometers, such as quadrupoles (5–10 spectra/sec) or ion traps (10–15 spectra/sec). Therefore, TOF-MS has improved sensitivity and detection limits. The ability to take many spectra per unit time offers another advantage in facilitating the deconvolution of mixed spectra that is resolving the MS data from one compound from a mixture of compounds that co-elute. If it is required to resolve one compound from another to obtain a MS identification TOF-MS with proper software is able to make identification and quantificatio frequently without the need for peak resolution [15,82]. TOF-MS has been widely used in fl vour analysis of foods, such as cooked beans [104], grains [105,106], wines [107,108], olive oil [109], roasted beef [110], and Cheddar cheese [111]. In the fiel of seafood, it has been mainly used for quality control (fis authentication by analysis of biomarkers) [112].

5.3.3 Electronic nose

The electronic nose (e-nose) offers a third technique (between instrumental and sensory analyses) for analyzing food aroma. E-nose is based on a process similar to the human olfactometry system in that both e-nose and human olfactory systems consist of an array of receptors (sensors), yielding a pattern (signal) of response to any given aroma. The brain, in the case of humans, and the computer, in the case of e-nose, make judgments based on a pattern recognition process as to the aroma and its quality [15].

   In e-nose, the sample is placed into a glass vessel. Transfer of the headspace vapour to the sensor array can be achieved either by diffusion or by pumping the vapour to the sensors. The sensors are key components of the e-nose system. Currently, there are several types of sensors, including semiconductor gas sensors, surface acoustic wave devices, biosensors/enzyme sensors (designed to measure a specifi compound), conducting polymer sensors, and mass spectrometry-based sensors [113,114]. In the case of an MS-based e-nose, the analyst can programme the system to detect some target sensory-relevant volatile components. The data taken from the e-nose is usually statistically analyzed using software in order to interpret the e-nose pattern towards the target analyte. Other detail reviews can be found elsewhere [113,114]. The e-nose has been widely employed in the quality control fiel of seafoods, such as detection of spoiling Alaska Pollack [115] and octopus [116].

5.4 Conclusions

There are numerous methods for the isolation and analysis of the volatile fl vour components of seafoods and seafood products. Among the various isolation techniques, headspace sampling methods are relatively simpler and faster than solvent or distillation extraction methods. Headspace methods also have advantages for the isolation of analytes with low molecular weights and high volatilities. However, for the exhaustive isolation of seafood fl vour compounds of intermediate and low volatilities, the later techniques are a better choice. In regard to the instrumental analysis of seafood fl vour, the classic tandem GC-MS based method is

predominant. Indeed, for certain specifi problems, alternative approaches may sometimes be superior. Consequently, with a problem as difficul and complex as studying and analyzing the fl vour components of seafood and its products, it is recommended to consider all possible techniques and procedures and choose those that are available and which might yield constructive information.

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6 Quality assessment of aquatic foods by machine vision, electronic nose, and electronic tongue

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6.1 Introduction

The increase in demand for seafood products has catalyzed the desire for higher standards regarding safety and quality issues. Since seafoods are perishable, freshness is a major quality parameter to be considered [1,2]. There is no unique freshness or spoilage indicator for seafood, therefore combinations of selected indicators need to be used to evaluate freshness [3,4]. An important and widely used method to determine freshness is sensory evaluation [5]. The Quality Index Method (QIM) uses a demerit point scoring system [6] based on the evaluation of the important sensory attributes (odour, texture, and appearance) of fis and other aquatic foods. The sensory quality is expressed by the sum of the demerit points, and a linear correlation between these points and the storage time is used to predict the freshness of the target seafood [5,7,8]. The QIM has been developed for various seafood species and products, such as Atlantic mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*), European sardine (*Sardina pilchardus*) [9], gilthead seabream (*Sparus aurata*) [10], farmed Atlantic salmon (*Salmo salar*) [11,12], and cod (*Gadus morhua*) [13], etc. Even though QIM is fast and reliable in determining the freshness of seafood, it still requires experts to evaluate the quality attributes. Alternatively, appearance, odour, and taste can be measured by machine vision system (MVS), electronic nose (e-nose), and electronic tongue (e-tongue), respectively.

In this chapter, the measurement of visual, odour, and taste quality of seafood using MVS, e-nose, and e-tongue is discussed. A few literature examples are given for all techniques, some of which are given from research conducted in our own laboratories.

6.2 Visual quality

Visual quality of seafood includes appearance (size, shape, and colour) attributes. These have a direct influenc on the seafood's value and acceptance. One of the methods of measuring them is by using a MVS, which consists of a digital camera to acquire images, an illumination system (e.g. a light box with fluorescen bulbs as lighting source), and computer software to analyze the image [14,15]. This is a rapid, objective, repeatable, and non-destructive method,

and has been recognized as the most promising approach to objective evaluation of visual quality of seafood, with many successful applications. For the industry, the implementation of an on-line inspection system can increase speed, efficien y, and accuracy along with cost reduction.

6.2.1 Visual quality determination based on size and shape

Fish is sorted according to species, size, and quality after harvesting. This is performed manually, and is a labour-intensive and expensive process. Sorting can be accomplished continuously, automatically, and reliably using MVS or computer vision system (CVS). Common carp (*Cyprinus carpio*), St. Peter's fis (*Oreochromis* spp.), and grey mullet (*Mugil cephalus*) have been successfully separated using images of fis swimming in an aquarium [16]. Besides sorting of fish CVS has been used to describe the rigor contractions of unstressed and stressed Atlantic salmon (*S. salar*) and Atlantic cod (*G. morhua*) fillet by monitoring the transient two- and three-dimensional changes in the geometry [17]. This method has been found suitable for industrial purposes. A method for quality grading of whole Atlantic salmon (*S. salar*) has also been developed using CVS [18], based on the external geometrical information from fis images.

Shrimp quality inspection relies on subjective sensory evaluation and routine sorting, counting, and weighing performed by trained inspectors. The uniformity ratio (UR) is calculated by taking the weight ratio of the largest 10% of shrimp to the smallest 10%. The inspectors determine visible defects (melanosis-black spots formed by the polyphenol oxidase enzyme), foreign materials, shell parts, and missing pieces (tails or segments). A MVS has been developed to determine the count and UR of whole, headless, peeled-tail-on, and peeled-tail-off white shrimp (*Paneus setiferus*) and headless, peeled-tail-on, and peeled-tail-off tiger shrimp (*Paneus monodon*) [19]. A similar experimental set-up has been used for whole, headless, peeled-tail-on, and peeled-tail-off white shrimp (*P. setiferus*) [20]. The authors concluded that this system could be used industrially if there were no shrimps touching or partially blocking each other.

Oysters are mostly sold by volume and grading is important for pricing. This is performed by humans and is labour-intensive and time-consuming. Predicting the volume or weight of oysters by MVS could be beneficial Several studies have been performed to sort and grade oyster meat with MVS [21] to predict the volume by using a laser-line based method, and to obtain the thickness information by the shape of the laser line on the meat [22]. The volumes (overall, shell, and meat) of oysters from Florida, Texas (*Crassostrea virginica*) and Alaska (*Crassostrea gigas*) have also been measured using the Archimedes principle. The top- and side-view images of whole oysters were captured by MVS and the actual view areas have been calculated by calibrating their pixel area with that of a known reference square. The view area information was used to predict whole oyster volume and weight, and meat volume and weight. The r^2 values for the predicted oyster volumes were 0.85, 0.92, and 0.64 for oysters from Florida, Texas, and Alaska, respectively [23].

6.2.2 Visual quality determination based on colour

Colour is determined by colorimeters, spectrophotometers, and MVS. During the last two decades, the popularity of MVS increased due to its advantages. It can measure the colour of a sample whether it is small or very large in size, and irregular in shape. For example, a shrimp may be too small to cover the viewing aperture of a colorimeter, or a salmon may be too

Fig. 6.1   Irradiated salmon colours measured by Minolta and machine vision system and their actual pictures. Adapted with permission from Yagiz *et al.* [26]. For a colour version of this figure, please see the colour plate section.

large to be measured all at once, requiring an average of several measurements to represent the actual salmon colour. These average L* (lightness/darkness), a* (redness/greenness), and b* (yellowness/blueness) values may not give the actual colour of the sample [15]. MVS can determine L*, a*, and b* values for each pixel of an image and analyze the entire surface of homogeneous and nonhomogeneous shapes and colours of samples. MVS also provides the colour spectrum and other visual attributes of the sample [24,25]. The performance of a hand-held Minolta colorimeter and a MVS in measuring the colour of Atlantic salmon (*S. salar*) fillet treated with different electron beam doses (0, 1, 1.5, 2, and 3 kGy) was compared [26]. The average L*, a*, and b* values measured by MVS resulted in orange colours very close to that of the original sample (Fig. 6.1). On the other hand, average L*, a*, and b* values measured by Minolta resulted in purplish colours. The standard red plate readings were similar for both systems. The reason for this difference is not known and needs to be investigated. The authors suggest visually comparing the average colours reported by any system against the actual sample colour.

Muscle colour is an important factor in consumer perception of meat quality [27]. Consumers mostly associate colour with freshness, better fl vour, and high product quality [28]. Processing techniques and packaging conditions affect seafood colour. High pressure processing could extend the shelf-life of seafood; however, this process causes a change in the colour of rainbow trout (*Oncorhynchus mykiss*) and mahi mahi (*Coryphaena hippurus*) [29]. The high pressure processing in combination with cooking treatment was also found to affect the colour of Atlantic salmon (*S. salar*) [30]. Changes in the colour of salmon fillet have also been investigated during thermal sterilization processes [31]. A CVS was used to determine accurate colour and to measure shrinkage. Colour of salmon fillet change during thermal processing, since heating denatures the myoglobin and oxidizes carotenoid pigments [32] in the muscle of salmon, and colour changes from red to pale pink, as reflecte in CIE L*, a*, and b* values. The colour of fresh tuna treated with gas (4% CO, 20% CO_2, and 10% O_2), irradiation (1 or 2 kGy), or combination of gas and irradiation has been evaluated with MVS [14]. The R (Red), a*, and hue parameters of the tuna samples have been measured. CO exposure increased the redness and preserved it during 12 days of storage at refrigerated temperatures. This is explained by the strong binding ability of CO to the haem in myglobin and haemoglobin to make it highly resistant to autoxidation and discolouration [33].

The diets used for fis feeding have an effect on the muscle colour. The impact of commercial diets on the muscle colour of cultured Gulf of Mexico sturgeon (*Ancipenser oxyrinchus desotoi*) has been investigated [34]. The L*, a*, and b* values of uncooked fillet stored for up to 15 days on ice have been measured using MVS. A colour difference in the fillet of sturgeon, which were fed with catfish hybrid bass, and trout diets, was found. A comparison of colour measurements of these fillet using a hand-held Minolta colorimeter versus MVS has also been reported [35]. Overall colour change is define as ΔE (Eqn. 6.1):

$$\Delta E = \sqrt{\left(L^* - L^*_{ref}\right)^2 + \left(a^* - a^*_{ref}\right)^2 + \left(b^* - b^*_{ref}\right)^2} \tag{6.1}$$

Colour change during storage is calculated by taking time zero colour values as reference (subscript $_{ref}$ in Eqn. 6.1). ΔE values were calculated using L*, a*, and b* values obtained from both devices. The respective ΔE values were significantl different between hand-held Minolta colorimeter and MVS at days 5, 10, and 15. Little colour change was observed over storage time using MVS and this was also observed visually in the images of the centre slices of the sturgeon fillets The MVS could easily determine the variability in colour within a fille surface. It was concluded that MVS provided valuable information regarding colour uniformity of a food product without increasing the number of readings required for each sample. MVS images could be kept in picture format and could be useful in automation and in-line quality control of food products' colour [35].

MVS could be used for automated quality control and grading of salmon fillet based on colour. The changes in skin and fille colour of anesthetized and exhausted Atlantic salmon after killing, during rigor mortis, and after seven days of ice storage have been investigated [36]. Atlantic salmon (*S. salar*) fillet have been sorted based on their colour obtained by CVS [37]. Human inspectors also evaluated the colours of fillet visually according to the Roche *Salmo*Fan$^{\text{TM}}$ lineal standard. No significan differences were observed between the CVS and inspections made by humans ($P < 0.05$). It was concluded that CVS could replace manual labour in fis processing companies.

Blood residues have a negative effect on the shelf-life, meat quality, and sensory attributes of fish Their impact on the quality of exsanguinated and unbled farmed trout (*Scophthalmus maximus*) was investigated [38]. Exsanguination improved the visual quality and CVS was able to quantify blood residues in the farmed trout. Other applications of the MVS to seafood quality evaluation have already been discussed [39].

6.3 Smell-related quality

Volatile compounds contributing to the characteristic odour of aquatic foods can be measured to determine their freshness [40]. Qualitative and quantitative analyses of volatiles of seafood products can be performed by using gas chromatography (GC) or gas chromatography/mass spectrometry (GC/MS). Individual components could also be correlated to sensorial perception using GC-olfactometry (GC-O) [41].

Fresh fis has no fis y odour, but smell develops with time after the fis is dead. Fish degradation after harvest is generally attributed to microbial spoilage, enzymatic degradation, and lipid oxidation. The composition of fis headspace, which is a result of microbiological and chemical degradation, gives information about its freshness [42,43]. Long-chain alcohols and carbonyl compounds, bromophenols, and *N*-cyclic compounds could be considered

as the major chemicals involved in the fresh fis odour. Short-chain carbonyl compounds, amines, sulphur compounds, aromatics, *N*-cyclic products, and acid compounds are produced upon microbial spoilage [40]. Definin fis freshness is a major problem, since the methods are time consuming, destructive, and labour-intensive [44]. Recent developments in sensor technologies and data analysis techniques have resulted in the development of rapid methods to detect post-mortem quality changes in foods [1,2,45]. Sensors for specifi gases have also been developed to detect trimethylamine (TMA) and dimethylamine (DMA), which are assumed to be the fis degradation products [46–50]. In fact, no single index can cover all the complex changes occurring during fis spoilage [51], thus multiple sensors could be used to perform simultaneous analysis of various sensory related attributes [2]. In this respect, e-noses based on selective detection of the important volatile compounds, which are contributing to the spoilage odour (i.e. amines, sulphur compounds, alcohols, aldehydes, and esters) could be used to rapidly determine quality changes in fis [52].

The concept of the artificia nose system was proposed in 1982 [53] and was called an "electronic nose" at the beginning of 1990s, define as "an instrument, which comprises an array of electronic chemical sensors with partial specificit , and an appropriate pattern-recognition system capable of recognizing simple or complex odours" [54]. The e-nose is composed of a sampling system, an array of gas sensors with different selectivities, a signal processing and conditioning system, and an appropriate pattern recognition algorithm to recognize simple or complex odours [54]. The most important part of an e-nose is the sensors. There are various types of sensors, and they need to be selected carefully to meet a particular application's requirements for precision, reproducibility, sensitivity, and stability, and to improve the discrimination characteristics of the aroma profiles In general, sensor types used in e-noses are metal oxide semiconductor (MOS), conducting polymer, surface acoustic wave (SAW), bulk acoustic wave (BAW) devices, metal oxide fiel effect transistors (MOSFET), electrochemical, smell-seeing, and GC/MS-based sensors [55,56].

The signals collected from the e-nose sensors are evaluated with appropriate pattern recognition techniques. Two basic approaches, multivariate data analysis and artificia neural networks, are commonly used. Principal component analysis (PCA), discriminant function analysis (DFA), cluster analysis (CA), partial least squares regression (PLSR), canonical correlation analysis (CCA), and fuzzy logic or artificia neural networks (ANN) are most frequently used as pattern recognition techniques [57–60].

Various gas sensors were used to detect fis freshness in the 1990s [2,46,61–63] and by the end of that decade, e-noses started to be used in assessing seafood quality [64,65]. Most of the studies on the use of e-noses to assess seafood quality during the last decade [44,66–96] are listed in Table 6.1.

6.4 Taste-related quality

One of the factors positively related to the consumption of seafood products is a liking for the taste of the product [97,98]. The sense of taste in mammalians is perceived by non-specifi taste buds, present on the papillae of the tongue. Overall, taste is correlated with a combination of basic tastes and taste sensations (bitterness, saltiness, sourness, sweetness, umami, metallic, astringency, spicy, and cooling effects). Interaction between different tastes may cause a desensitizing effect or threshold increase when two substances eliciting different tastes are present simultaneously. In addition to this, the decrease in sensitivity threshold

Table 6.1 Electronic nose applications to aquatic foods, with species, sensor types, and data analyses in the last decade

Product	Electronic nose used	Sensors	Data analysis techniques	Reference
European sea bass (Dicentrarchus labrax)	PEN2 model-Win Muster Airsense Analytic Inc. (Germany)	MOS	PCA and CA	[44]
Octopus (Octopus vulgaris)	Custom-made portable electronic nose (China)	TGS	PCA and DFA	[66]
Moroccan sardines (Sardinia pilchardus)	Custom-made portable electronic nose (Morocco)	TGS	PCA	[67]
Blue crab (Callinectes sapidus)	Cyranose 320™, Cyrano Sciences Inc. (USA)	CP	PCA, CDA, and SDA	[68]
Smoked salmon (Salmo salar)	FishNose (Iceland)	MOS	PCA and PLSR	[69]
Sardines (Sardinia pilchardus)	Custom-made portable electronic nose (Morocco)	MOS (TGS)	PCA, DFA, and FANN	[70]
Pink shrimp (Pandalus jordani)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[71]
Cod (Gadus morhua)	FreshSense, Maritech (Iceland)	ECS (CO, H$_2$S, SO$_2$, and NH$_3$)	PCA and PLSR	[52]
Smoked Atlantic salmon (Salmo salar)	FishNose, Optotek Engineering (Slovenia) (adapted from GEMINI e nose-Alpha MOS, France)	MOS	PCA and PLSR	[72]
Alaska pink salmon (Oncorhynchus gorbuscha)	Cyranose 320™, Cyrano Sciences Inc. (USA)	CP sensors	PCA and FSGDA	[73]
Haddock (Melanogrammus aeglefinus)	FreshSense, Icelandic Fisheries Laboratories and Maritech (Iceland)	ECS (CO, H$_2$S, SO$_2$, and NH$_3$)	PLSR	[74]
Sardines (Sardinia pilchardus)	EnQbe, Tor Vergata (University of Rome and CNR (Italy)	TSM resonators	PLSR	[75]
Alaska pink salmon (Oncorhynchus gorbuscha)	Cyranose 320™, Cyrano Sciences Inc. (USA)	CP sensors	PCA and FSGDA	[76]
Smoked Atlantic salmon (Salmo salar)	Gemini, Alpha MOS (France) FishNose, Optotek (Slovenia)	MOS	PCA and PLSR	[77]
Shrimp (Pandalus borealis)	FreshSense, Maritech (Iceland)	ECS (CO, H$_2$S, SO$_2$, and NH$_3$)	PCA	[78]
Cod (Gadus morhua)	FreshSense, Bodvaki-Maritech (Iceland)	ECS (CO, H$_2$S, SO$_2$, and NH$_3$)	ANOVA	[79]
Baltic cod (Gadus morhua)	NST 3320, Applied Sensor (Sweden)	FE, MOS	PCA and regression	[80]

(Continued)

Table 6.1 (Continued)

Product	Electronic nose used	Sensors	Data analysis techniques	Reference
Anchovy (*Engraulis japonica*) sauce	e-NOSE 4000, Neotronics (UK)	CP sensors	PCA	[81]
Capelin (*Mallotus villosus*)	FreshSense, IFL, Element Sensor Systems (Iceland)	ECS (CO, H_2S, SO_2, and NH_3)	ANOVA	[82]
Cod (*Gadus morhua*) roe	FreshSense, IFL, Bodvaki-Maritech (Iceland)	ECS (CO, H_2S, SO_2, and NH_3)	PCA	[83]
Herring (*Clupea harengus*)	Custom-made system (Norway)	MOSFET, TGS	PLSR	[84]
Anchovy (*Engraulis encrasicholus* L.)	AromaScan, AromaScan Inc. (UK)	CP sensors	PCA and ANN	[85]
Atlantic salmon (*Salmo salar*)	AromaScanTM, AromaScan Inc. (USA)	CP sensors	DFA	[86]
Atlantic salmon (*Salmo salar*) Haddock (*Melanogrammus aeglefinus*) Atlantic cod (*Gadus morhua*)	Custom-made system (USA)	MOS	NN	[50]
Redfish (*Sebastes marinus*)	FreshSense, Bodvaki Company (Iceland)	ECS (CO, H_2S, SO_2, and NH_3)	PCA	[87]
Tilapia (*Oreochromis niloticus*)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[88]
Catfish (*Ictalurus punctatus*)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[89]
Argentinean hake (*Merluccius hubbsi*)	Custom-made system (Argentina)	Polycrystalline tin dioxide sensors	PCA	[90]
Atlantic cod (*Gadus morhua*)	FreshSense, Element Bodvaki Company (Iceland) LibraNose, Tor Vergata University of Rome with Technobiochip Company (Italy)	ECS (CO, H_2S, NO, SO_2, and NH_3) TSM resonators	PLS-DA	[91]
Mahi-mahi (*Coryphaena hippurus*)	AromaScan, AromaScan Inc. (USA)	CP sensors	DFA	[92]
Capelin (*Mallotus japonica*)	FreshSense, IFL, Element Sensor System (Iceland)	ECS (CO, H_2S, NO, SO_2, NH_3A7AM, and NH_3)	PCA and PLSR	[93]
Shrimp (*Penaeus aztecus, Litopenaeus vannamei,* and *Penaeus monodon*)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[94]
Atlantic salmon (*Salmo salar*)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[95]
Yellowfin Tuna (*Thunnus albacares*)	e-NOSE 4000, EEV Inc. (UK)	CP sensors	DFA	[96]

Abbreviations: ANN, Artificial neural network; CA, Cluster analysis; CDA, Canonical discriminant analysis; CP, Conducting polymer; DFA, Discriminant function analysis; ECS Electrochemical sensors; FANN, Fuzzy ARTMAP neural networks; FE, Field effect sensor; FSGDA, forward stepwise general discriminant analysis; IFL, Icelandic Fisheries Laboratories. MOS, Metal oxide semiconductor sensor; NN, Neural network; PCA, Principle component analysis; PLS-DA, partial least significant discriminant analysis; PLSR, Partial least squares regression; SDA, Stepwise discriminant analysis; TGS, Taguchi gas sensor.

may occur when substances are present at non-perceptible concentrations. In fact, perception thresholds of the human tongue to most tastes are much higher compared to those for olfaction [99].

In the last decade, a novel instrument, the e-tongue, has been developed to detect the tastes of food samples, especially liquid samples. This instrument is composed of a sensor array in combination with pattern recognition tools [100,101]. Most of the e-tongues reported so far consist of a combination of electrochemical methods based on potentiometric [102] or amperometric sensors [103]. These instruments have been widely used in quantitative analyses of liquids such as milk [104], alcoholic drinks (beer and wines) [102,105–107], vegetable and olive oils [108–109], natural and mineral waters [104,110,111], and various fruit juices [112,113], etc. The e-tongue has been applied to determine fis freshness [114, 115]. Simple Au and Ag wires have been used for the analysis of minced gilthead sea bream (*S. aurata*) and it has been found that this method could be used for the evaluation of fis freshness [114]. However, the e-tongue has limited application in the seafood area since the sample needs to be minced for e-tongue application. The e-tongues are more for use in determining the taste properties of liquid food products.

6.5   Combination of machine vision system and electronic nose

The MVS and e-nose combinations offer possibilities for the development of accurate and rapid measurement of quality of seafood in "orthogonal" dimensions. Therefore, their combination offers increased resolution of the discrimination capacity compared to the methods considered individually. The ability of e-nose and MVS to classify tilapia fillet based on their odours and colour has been investigated [88]. When e-nose data together with machine vision data were used to analyze the quality of tilapia fillets the classificatio rates were higher than analyses using either data alone. Similar results were found when e-nose data alone were used for analyzing the quality of raw and cooked catfis fillets The correct classificatio rates were lower than the ones obtained by using MVS together with e-nose [89]. These results point towards the advantages of the discriminating ability based on two independent quality parameters (colour and odour) considered together.

6.6   Conclusions

Rapid, objective, and non-destructive determination of smell and visual attributes of foods is possible with MVS and e-noses. Their combination increases the resolution of the discrimination capability of the analyses. MVS can be calibrated to assure comparability of images and results from different laboratories. E-noses have slightly different sensors between instruments, and their calibration is more challenging for seamless exchange of data between laboratories. For their widespread application certifie data bases need to be developed, possibly with information regarding the important odour-active components of the sample atmospheres. Battery-operated portable e-noses can make fiel and on-line plant applications possible. The digital and discrete nature of the data from MVS and e-noses allow new possibilities for traceability and documentation of the quality of aquatic products.

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7 Effects of nutrition and aquaculture practices on fish quality

Kriton Grigorakis

7.1 Introduction

The term fis quality is define by a group of various factors. These include:

1) appearance, shape, size, and external look (colour, malformations, and injuries);
2) nutritional value;
3) fat deposition;
4) organoleptic characteristics (odour, taste, and texture);
5) freshness; and
6) filletin yield.

It is often difficul to outline the way that quality is affected by feeding, due to numerous endogenous and exogenous factors that influenc quality simultaneously. In the endogenous factors, size, sex, stage of life cycle, and genetic factors are included. The exogenous factors include feeding, fis population crowding, temperature, salinity, physical exercise of the fish and sources of external stress. A multifactorial analysis including nutritional and environmental parameters showed clearly that the effects of feeding on fis quality strongly depended on the environmental factors and that the interaction of feeding and environment actually define the fina results [1]. Despite the complicated interactions, the ability still exists to relate feeding to the produced quality and at the next level to tailor quality through feeding. This chapter attempts to outline the effects of feeding and aquaculture handling on fis quality and to examine to what extent the quality of the end-product can be manipulated.

7.2 The role of muscle composition and fat deposition in fish quality

The edible part of the fis is actually the fillet Therefore, the fille composition is what define the fis quality. Fish store energy as fat to be utilised when needed. Fat is abundant
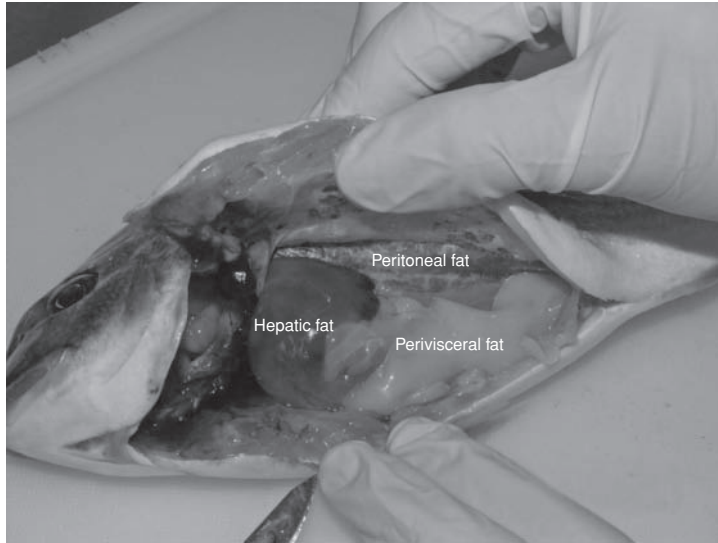
Fig. 7.1   Anatomy of gilthead sea bream – forms of deposited fat, excluding muscle fat that is not visible. For a colour version of this figure, please see the colour plate section.

in four deposited forms:

1) the liver or hepatic fat;
2) the muscle fat;
3) the perivisceral fat (also termed as visceral fat or mesenteric fat); and
4) the peritoneal fat, deposited around the peritoneum (Fig. 7.1).

The distribution of these fat deposits mainly depends on the fish species. The three forms of fat (muscle, perivisceral, and peritoneal) are important in terms of fish quality. The role of these fat deposits in fish quality is summarised in Table 7.1. Muscle protein content may not have the prime importance of fat, but also contributes to the sensory quality through water-interacting proteins. Also, in cases of long-term fasting, when losses of muscle protein occur, the cooked flesh becomes soft with reduced cohesiveness [2]. Another important quality parameter with respect to the nutritional value of proteins is the amino acid composition and, in particular, the essential to total amino acids ratio. Non-protein nitrogen (NPN) is another important qualitative determinant in fish NPN contributes 9 to 18% of the total nitrogen in teleost fish It mainly consists of volatile bases, creatine, creatinine, free amino acids, nucleotides, and purines. Volatile nitrogen plays an important role in the organoleptic properties of the fish since it contributes to its flavour and odour [3].

7.3   Effect of feeding and aquaculture practices on quality characteristics

7.3.1   Feeding and its impact on fish fat

Feeding has a key role in the quality of the aquacultured fish Generally, fat deposits increase with weight irrespective of the feeding; for example, larger fish tend to have higher lipid

Table 7.1 Roles of fat deposits in fish quality

Fat form	Quality attribute	Role of fat in quality
Muscle fat	Taste	Fats have slight taste themselves.
	Flavour	Lipid-derived volatile compounds characterise fish flavour and spoilage off-flavour.
	Mouth sensation	Tissue becomes softer, fattier, and juicier when fat increases. A 1–2% fat increase drastically changes the quality on non-fatty fish (e.g. halibut), while in fatty species (e.g. salmon) it has negligible impact.
	Texture	Decrease of firmness when fat increases.
	Nutritional value	Increase of PUFA contents is related to health benefits: reduction of heart diseases and inflammatory diseases, contribution against some forms of cancer, and significant role in embryonic brain development.
Perivisceral fat	Visual sense	Increased quantity negatively affects consumers' impression about the fish when fish commercialised as whole ungutted.
	Smell	Characteristic, strong, and not pleasant smell of perivisceral fat.
Peritoneal fat	Taste or flavour	Unknown impact, since this fat form is consumed together with the fish fillet.

content. This has already been shown for gilthead sea bream [4], eel [5], catfis species [6], carp [7], and salmonids [8–10].

7.3.1.1 *Feeding intensity and dietary fat*

The seasonal differences in fis fat deposits in Nature occur, beyond the sexual maturation process, due to different feeding intensity throughout the year. In feeding intensity, seasonal differences exist because of lower food availability in the cold months and also due to reduced metabolism when water temperatures decrease. This metabolism reduction is confirmed even for intensively aquacultured fish where feed availability is not an issue.

The impact of feeding on fat deposits depends, to a large extend, on the species of interest (Table 7.2). Thus, there is a preferable deposition in the muscle fat for certain species, such as flatfi [13,34], while other species, such as perch, preferably accumulate fat in the peritoneum [28]. Some species also follow a completely different pattern of fat deposition, such as cod that tend to accumulate the dietary fat almost exclusively as liver fat [26,35], and the adult eel, where feeding seems to have almost negligible impact because main muscle fat deposition occurs earlier during its development [5]. However, contradictory results have been found, even within the same species, with respect to the effects of feeding rate, dietary lipids, and starvation, in the fis fat deposits. The main reason is the variability of the experimental conditions. Most studies focus on the results of a dietary treatment under specifi or stable environments and in these cases the environmental impact is not obvious.

In most cases, increase of feeding rate and dietary fat leads to increased muscle and perivisceral fat. In salmonids, it was shown that leaner fis were obtained when the feeding rate was reduced, even when referring to high fat feeds [9,18]. This indicates that the feeding rate in salmonids plays a more important role in fat deposition than the dietary fat.

Each aquaculture fis species has its own energy needs and thus its own dietary lipid needs. When dietary lipids exceed these limits, the result is an excessive fat deposition.

Table 7.2 Effects of various feeding treatment on the fat depots of various aquacultured fish species. All effects refer to the end-product quality

Species	Feeding manipulation	Effects on muscle fat	Effects on visceral/peritoneal fat	Remarks	Reference
Gilthead sea bream (Sparus aurata)	Increase of dietary fat	Muscle fat unchanged	Increase of perivisceral and peritoneal fat	Ad libitum feeding, dietary fat 10–20%	[11]
	Increase of feeding ratio	Increase of muscle fat			[11,12]
	Comparative feeding with pelleted and extruded diet	Increased muscle fat in fish received the extruded diet		Extruded diet with lower fat levels	[13]
	3-week starvation	Decrease of muscle fat	No reduction of perivisceral fat		[4]
Sea bass (Dicentrarchus labrax)	Increase of dietary fat	Increase of muscle fat	Increase of perivisceral and peritoneal fat	Dietary fat 11–19%	[14]
	60 days starvation		Reduction of perivisceral fat		[15]
Sunshine bass (Morone chrysops x M. saxatilis)	Increase of dietary fat	Small increase of muscle fat	Increase of perivisceral fat	Dietary fat 9–17%	[16]
Atlantic salmon (Salmo salar)	Increase of dietary fat	Increase of muscle fat or negligible effect	Higher increase of perivisceral fat	Dietary fat 21–30%	[17,18]
	Increase of feeding ratio	Significant increase of muscle fat	No effect (increase in other cases)		[10,19]
	Increase of dietary carbohydrates	Increase of muscle fat	Increase of perivisceral fat	For given dietary fat level	[20]
	3–86 days starvation		No effect		[19]
Pacific salmon (Oncorhynchus tshawytscha)	Increase of dietary fat	Increase of muscle fat	Increase of deposit fat		[21]
	Increase of feeding ratio	No effect	No effect		[22]

(Continued)

Table 7.2 (Continued)

Species	Feeding manipulation	Effects on muscle fat	Effects on visceral/peritoneal fat	Remarks	Reference
Rainbow trout (Oncorhynchus mykiss)	Increase of dietary lipid + increase of its feeding duration	Increase of muscle fat	Increase of perivisceral fat (higher than muscle fat)	Dietary fat 19–31%	[23]
	Increase of feeding ratio		No effect in perivisceral fact		[9]
	61 days starvation	Intensive decrease of muscle fat	Moderate decrease of perivisceral fat		[23]
	2–3 months starvation	Small decrease	decrease of perivisceral fat		[9]
Brown trout (Salmo trutta)	Increase of dietary fat	Increase of muscle fat (higher than in perivisceral fat)	Increase in perivisceral fat	Triploid fish Dietary fat 11–26%	[24]
	8 weeks starvation	Decrease of muscle fat	Perivisceral fat unchanged	Triploid fish	[24]
Atlantic halibut (Hippoglossus hippoglossus L.)	Increase of dietary fat	Higher increase of muscle fat	Increase of perivisceral fat		[25]
	Increase of pellet size	Small increase of muscle fat		Pellet diameters 16–27 mm	[25]
Cod (Gadus morhua)	Increase of dietary fat	Negligible effect	Negligible effect	Increase of liver fat	[26]
White fish (Coregonus lavaretus)	Increase of dietary fat	Increase of muscle fat	Increase of perivisceral fat	Dietary fat 12 and 27.5%	[27]
Eurasian perch (Perca fluviatilis)	Increase of dietary fat	Minimum effect in muscle fat	Mainly increase of perivisceral fat		[28]
European eel (Anguilla anguilla)	Increase of dietary fat	No alteration in fat depots			[5]

Species	Treatment	Effect on muscle fat	Effect on perivisceral/deposit fat	Notes	Reference
Carp (*Cyprinus carpio*)	Increase of dietary fat	Increase of muscle fat	Increase of perivisceral fat		[7]
	Increase of feeding rate	Increase of muscle fat	Increase of perivisceral fat		[7]
	Starvation	Decrease of muscle fat			[29]
European catfish (*Silurus glanis*)	Natural feeding vs. industrial feed	Higher muscle fat in fish receive industrial diet	No alteration in perivisceral fat	Feeding *ad libitum*, in tanks.	[30]
Channel catfish (*Ictalurus punctatus*)	Increase of dietary fat	Increase of muscle fat	Increase of muscle fat		[6]
	Increase of feeding rate	Increase of muscle fat	Increase of total deposit fat	Restricted feeding vs. *ad libitum*	[3]
African catfish (*Clarias gariepinus*)	Increase of dietary fat	Muscle fat unchanged	Increase of perivisceral fat	Dietary fat 8 and 13%	[32]
Blue catfish (*Ictalurus furcatus*)	Increase of dietary protein from 27–36%	Significant increase of muscle fat	Unchanged perivisceral fat		[33]

Thus, in gilthead sea bream, where the optimum dietary fat level is 15%, feeds with 20% lipid significantl increase fat deposition. Sea bass, on the other hand, exhibiting a more carnivorous feeding behaviour, is better adapted to the higher fat feeds and has much better quality characteristics when receiving them [36]. The salmonids, in general, are better accustomed to feeds with even higher lipid contents, where dietary fat reaches 30% [9].

Limited information is available on the exact impact of various protein levels and of dietary carbohydrates in fis quality. However, the optimum protein levels for achieving a good quality product depend on the fis needs in the way they are dictated by their nature. These requirements are lower for herbivorous and omnivorous species and higher for carnivorous species. In general, increase of dietary protein in isoenergetic diets (e.g. same lipid) often leads to a higher muscle fat [28]. With respect to carbohydrates, fis have negligible requirements, with few exceptions. In feeds, carbohydrates are used as low-cost energy sources and as binders to ensure cohesion of the feed pellet in the water. Increase of digestible dietary carbohydrates in the diet leads to a higher fat deposition [7,20].

There are indications that dietary treatments may also affect the fatty acid composition of the muscle. A well-established rule is that muscle fatty acids of the fis reflec the dietary fatty acids, and this has been confirme for most of the aquacultured fis species, including salmonids [9,37], Mediterranean species [36,38], carps [7], cod [35], catfis species [6], and flatfi [34]. Therefore, manipulation of the fatty acid profil of the end-product can be achieved.

In salmonids, there were cases that showed a decrease of docosahexaenoic acid (DHA, 22:6 n-3) and omega-3 polyunsaturated fatty acids (PUFA), and a respective increase of monounsaturated fatty acids (MUFA) when feeding rate increased [8]. In gilthead sea bream and sea bass, there were indications of positive correlation between dietary fat and the omega-3 levels [36].

7.3.1.2 *Fish oil substitution*

Due to the sustainability issues that the use of fis oils raises, there is a turn towards the use of plant oils in fis feeds. The result of dietary fish-oi substitution by plant oils is a change in fis fatty acid composition. In all cases, the most profound alterations in fis muscle are the decrease of eicosapentaenoic acid (EPA, 20:5 n-3) and DHA in net quantities, as well as the decrease of EPA/DHA and n-3/n-6 ratios [35,38]. Reduction of arachidonic acid (ARA, 20:4 n-6) was also observed in the cases of experimental substitution with soybean oil, rapeseed oil, sunfl wer oil, and linseed oil, but not in the case of substitution with olive oil [39,40]. The magnitude of these changes depends on the feeding period and the degree of the fis oil substitution [40–42].

7.3.1.3 *Finishing diets*

Part of the research also focuses on the effects of the finishin diets on fis quality. Thus, re-feeding fis that previously received plant oils with diets containing fis oil retrieves, up to a great extent, the initial fatty acid profile It is shown that a feeding period of 90 days with a fis oil-containing diet is adequate to restore almost fully the initial muscle fatty acids in both gilthead sea bream and sea bass. However, EPA cannot be recovered, even within a longer period (150 days) [40,41]. However, in red sea bream (*Pagrus auratus*), linoleic acid (18:2 n-6) is the fatty acid that is not easily restored [38]. Atlantic salmon that previously received vegetable oils fully restored the omega-3 PUFA when fed with fish-oi finishin

diets [43]. Finishing diets have also been effectively used for freshwater aquacultured fis such as carp and tench (*Tinca tinca*) [44]. Beyond the inter-species differences, the recovery changes depend on the plant oil that has been previously used. Thus, red sea bream that was previously fed on soybean-oil retrieved EPA quicker than fis previously fed on canola oil [38]. Finally, there are indications that fatty acid recovery also depends on fis size and that larger fis tend to have slower fatty acids recovery [38].

7.3.1.4   *Fasting*

During fasting, there are different fat deposits mobilisation patterns in different species (Table 7.2). Water temperature also seems to influenc the fasting effects for most of the aquacultured species [9,15]. In addition, Rasmussen [9] has noted that mobilisation is dependent upon the genetic pool of the fish fis density, and water salinity. The previous feeding history also seems to be important in determining the fasting impact [4,9]. Finally, the magnitude of the impact is found to depend on the duration of fasting. Usually, in custom aquacultural conditions, food deprivation occurs only for a few days, aiming at emptying the fis intestines, and has no effect in the fis fat depots. Furthermore, fasting can affect the muscle fatty acids. Thus, in some cases (e.g rainbow trout), a reduction in the relative MUFA is observed, while in others (e.g Atlantic salmon) there is an increase or no impact in the MUFA level [9]. In farmed sea bass, a two-month food deprivation showed reduction of saturated fatty acids (SFA), especially 17:0, and retained the total PUFA, but was accompanied with a reduction in EPA, as well as formation of 20:2 n-6 as a 18:2 n-6 elongation product [45].

7.3.1.5   *Factors other than feeding that affect fish fat*

Since most of the existing research focuses on feeding, less data are available on other factors that may be employed to manipulate fis fat. Genetic predetermination in fis fat deposition has been shown in various cases [6,9]. The degree of exercise seems also to be a determinant of muscle fat in salmonids [9]. Moreover, environmental factors including salinity and water temperature are found to influenc both fat deposition and fatty acid composition [46].

7.3.2   Feeding and handling: effect on muscle protein/amino acids

Under normal situations, the levels of muscle protein remain unchanged in organisms that have completed their development (adults). With respect to the amino acid composition, factors such as salinity and season seem to exert an effect [36]. Contradictory data about the effect of feeding on muscle amino acids have appeared in salmonids [47], but not in other species such as carp [7].

7.3.3   Feeding and aquaculture handling: effects on colour

The colouration of skin and fles is clearly related to the feeding of the organism. Thus, carotenoid intake, to a great extent, define the colour of the fillet Astaxanthin and castaxanthin absorption in salmonids and the dietary factors that affect it have been reviewed in the literature [37,48]. Mathematical modelling of salmon pigmentation also occurs [49].

Beyond the dietary carotenoids, the colour of fis fille is related to feeding due to the impact of the fat content. Elevated muscle fat is accompanied by a more whitish appearance

of the flesh Flesh colour is also related to aquaculture practices and specificall the rearing temperature [50]. External colouration of the fis depends on fis feeding practices. Thus, the external colouration of gilthead sea bream can be manipulated through various dietary carotenoid sources [51], and has also been noted to alter with fasting [4]. One of the most important problems in external colouration is that of red porgy (*Pagrus pagrus*), which under aquaculture conditions become grey. Although the exact nature of the problem is still unknown, an improved colouration has been achieved through dietary carotenoids [52].

7.3.4   Feeding and body shape

Beyond colouration, feeding also affects the body shape of the fish In Atlantic salmon, rainbow trout, and gilthead sea bream, reduced feeding rates or fasting result in a more spindle-shaped body [4,19]. However, the body shape seems rather genetically predetermined than regulated by feeding. Thus, geometrical modelling in gilthead sea bream shows that fis derived from different hatcheries and therefore genetically different, can be distinguished based on their morphometry, even if they have been raised at the same farms and under identical feeding conditions [53].

7.3.5   Feeding and effect on taste and flavour

Besides the impact of feeding on fille fat, which in turn affects its taste and fl vour, the effects of feeding in the formation of taste and fl vour is not very distinct. Most existing studies do not fin any quality differences between fis that have been fed with diets containing various fis meal substitutes, mostly of plant origin. However, there are some exceptions, where organoleptic differences have been found in gilthead sea bream that have received diets with soy meal at high substitution levels. The latter has been found to have a less pronounced "seaweed" fl vour than the fis receiving fis meal diets. These differences can possibly be justifie as free amino acid differences or generally as NPN differences [42].

Organoleptic differences often occur between fis that have received different fis oils or fis receiving different plant oils. These differences are more pronounced for higher plant oil inclusion levels [54]. This is due to the different fatty acids of the diets, since fatty acids are primers for a large number of volatiles characterising fis fl vour [55]. A direct correlation of dietary lipids [56], or dietary sulphur-containing amino acids [57], to the fl vour compounds of the fis seems to occur in some cases. However, to what extent a dietary control can be achieved remains unknown.

7.3.6   Dietary and handling impacts on texture

As already mentioned, muscle fat increase results in a softer and less fir texture [2,36]. There are observations in salmonids, positively relating hypertrophy (diameter increase) and hyperplasia (number increase) of muscle fibr to the growth rate [9]. Since the number and distribution of muscle fibre define the texture and in particular the hardness of muscle [3], any treatment that affects the growth rate can have an impact on muscle texture and this has been confirme for salmon [58]. However, some contradicting results also exist, such as those in Atlantic halibut, where no dietary impact on the distribution and generation of muscle fibre was found [34].

Starvation seems to be the treatment with the most pronounced impact on fis muscle texture. In some cases, short-time fasting improves the texture, producing a firme muscle

[9], while in other cases reduction of feeding or prolonged starvation results in reduced firmnes and increase of moistness and sweating of muscle [2,24]. Besides the dietary effects, rearing temperatures have been found to affect textural characteristics, with softer and less elastic fles observed for fis reared at lower temperatures [50].

7.3.7   Impact of aquaculture handling and killing procedure on post-mortem quality

Seasonality has been observed with respect to the post-mortem quality of fis [59,60]. In general, temperate-water fis in summer appear to have a slightly better quality. A possible explanation could be that summer elevated temperatures can result in a longer microbial lag phase due to the stronger thermal shock of the microflor when placed in ice. Further to the seasonality, the life history of the fis seems to play an important role in the post-mortem quality; a direct relationship of culture conditions to fis freshness has been indicated [12].

Both the pre-slaughter conditions and the killing procedures affect the post-mortem quality of fish Crowding stress results in longer struggling during killing, earlier rigor mortis onset, and more intense and shorter rigor mortis [61,62]. The impact of the killing method on the post-mortem quality is related to the amount of stress the fis receives. A stressful killing method, such as asphyxia, usually results in reduced post-mortem quality, organoleptically expressed as a low fl vour score [59]. Most of the killing procedures tested fail to show any particular quality advantage [9,63]. However, there are indications that rapid killing methodologies, such as spiking the brain, can result in longer shelf-life in some cases [63]. Some results for Mediterranean fis species show improved quality with slurry ice-killing over the classic ice-killing [64], while others fin practically no difference [65]. However, cloudy eyes have been mentioned as a negative impact of the use of slurry ice [65].

7.3.8   Effect of feeding on post-mortem quality and technological properties

An important feeding factor that can affect the post-mortem quality of fis is the dietary fatty acids, since PUFA are more susceptible to lipid oxidation. Thus, rancidity can be more pronounced for fis that have been fed higher levels of omega-3 fatty acids [66]. A dietary treatment with the aim of improving post-mortem quality is the use of various antioxidants in the diet, such as tocopherols and astaxanthine. Although results are generally contradictory, a better oxidative stability has been observed in fis receiving high dietary vitamin E [66]. There are indications that short-time fasting, applied in the aquaculture practice (1–2 days), leads to better preservation due to reduction of peptic enzymes in the intestine and consequently slower autolytic action [67].

The impact of feeding on fles lipid also affects the ability to fille and to process the fis thermally. In salmon, increase of the feeding rate and dietary fat induces the post-mortem gaping of the fis fille [9,19,68]. Fish that have received diets with soybean oil at high substitution levels show less gaping than fis receiving fis oils [35]. The fille gaping is a serious technological problem, especially in smoked fille production, and it has been related to low post-mortem pH and its impact on the connective tissues [2]. Therefore, it is directly related to the nutritional status of the fish but also to the stress the fis might have received prior to killing and depletion of the glycogen reserves [9].

7.4 Conclusions

Although the relationships between feeding and quality are complicated due to the impact of extrinsic factors, some manipulation of the end-product quality can be achieved though feeding and handling. Thus, fat deposition in the fille can be regulated through dietary lipid, feeding ration size, and fasting. The most profound regulation that can be achieved through diet is that of the muscle fatty acids. Beyond these, skin and muscle colouration can be manipulated through the intake of dietary carotenoids. Post-mortem quality of the fis is highly affected by the amount of stress the fis receives through handling and during killing.

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8 Lipid oxidation, odour, and colour of fish flesh

Jeong-Ho Sohn and Toshiaki Ohshima

8.1 Introduction

Lipid oxidation is the reaction between unsaturated lipids and molecular oxygen to form lipid hydroperoxides. The oxidation of unsaturated fatty acids is one of the most fundamental reactions in lipid chemistry. Lipid hydroperoxides have been identifie as the autoxidation products of polyunsaturated fatty acids (PUFA), and as the first-oxidatio products in lipid deterioration they lead to further oxidation. Hydroperoxides are unstable and break down to produce a myriad of secondary reaction products [1]. The secondary oxidation products, including aldehydes, ketones, free fatty acids (FFA), and alcohols, etc., are responsible for impairment of taste, fl vour, and texture in foods as well as a number of deleterious reactions in biological tissues. The oxidation of lipids not only induces the deterioration of the fles and the freshness of foods by the formation of secondary products during processing and storage, but is also involved in the biological damage implicit in a variety of diseases such as atherosclerosis, cancer, and rheumatoid arthritis, as well as in the process of aging [2–6]. Recently, an increase in the dietary intake of long-chain omega-3 (n-3 or ω-3) PUFA, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been shown to be beneficia in a variety of human disorders, including cardiovascular and inflammator conditions, and much research has been devoted to the biological importance of PUFA in terms of their physiological function [7–9]. Partially purifie EPA and DHA from fis oils are used as ingredients in both functional foods [10] and pharmaceuticals [11,12]. However, the high degree of unsaturation also renders the PUFA in fis fles highly susceptible to oxidation, even at low temperatures. Thus, lipid oxidation is the most critical parameter affecting the shelf-life of fis flesh

A wide range of secondary oxidation products, the low-molecular-weight compounds with strong olfactory attributes, impart the characteristic and disagreeable fl vour of rancid fis oil. The formation of unpleasant fl vour notes in fis oil during storage has also been satisfactorily modelled on the basis of the concentrations of only a few potent fl vour compounds [13,14]. Carbonyl groups affect the colour of food by turning it brown through the Maillard reaction. Hydroxy acids generated in the process of hydroperoxide oxidation are responsible for a bitter taste. In addition, aldehydes or radical groups transform protein and thus the physical properties of food and lead to a reduction in the nutritional value by oxidizing the vitamins

present, as well as the carotenoid pigments. The haemoproteins are also converted to met-protein, thus affecting the colour of the meat. The cholesterol oxides produced as a result of the accompanying oxidation in fis meat are risk factors for atherosclerosis and cancer. In addition, most of the secondary products of oxidation of PUFA, such as malonaldehyde, are strong mutagens. A series of chemical reactions of unsaturated fatty acids relate to lipid peroxidation. Therefore, these have a great influence not only on the quality of food in terms of smell, taste, colour, physical properties, and nutrition, but also on the safety of products. Quality degradation due to oxidation of fatty matters and its prevention deserve individual attention. Fish fles and foodstuffs containing fis oils are particularly susceptible to oxidative reactions due to their high PUFA contents. This chapter summarizes the mechanism of lipid oxidation of raw fis fles during the early stage of storage and explains effects on odour and colour of fis flesh

8.2 Quantitative determination methodology of total lipid hydroperoxides by a flow injection analysis system

Although various methods for the measurement of hydroperoxides as lipid oxidation indices have been proposed, instability and diversity of hydroperoxides in a complex food system hinders accurate and simple analysis. The widely accepted iodometric titration [15,16] and the enzymatic assays [17] have inherent problems such as sensitivity, selectivity, and inter-ference with contaminations. It is very important and therefore necessary to develop more efficien analytical methods to evaluate lipid peroxidation in complex food systems. In the analysis of hydroperoxides by high-performance liquid chromatography (HPLC), quantita-tive determination by ultra-violet (UV) light absorption at around 235 nm is based on the oxidation reaction of the conjugated double bonds (e.g. conjugated dienes, CD) of lipid molecules [18]. However, with respect to the applications to evaluate early stages of lipid ox-idation, the problems involve specificit and detection sensitivity. Thus, an electrochemical detection method that uses the redox potential of hydroperoxides to detect the primary lipid peroxidation product may be employed [19]. The detection sensitivity is, however, inade-quate to analyze trace amounts of lipid hydroperoxide. One solution to this problem involves post-column chemiluminescence detection based on luminol or isoluminol oxidation during the reaction of hydroperoxide and cytochrom C [20,21]. Some applications of the method-ology to biological systems have also been reported [22–24]. Generally, non-polar solvent systems based on *n*-hexane are used in HPLC for the separation of positional isomers of hydroperoxide [25]. However, a post-column reaction of luminol to luminol oxide progresses only under aqueous alkaline conditions [23]. Therefore, the number and types of organic solvents used for the mobile phase are relatively limited, since hydrophobic non-polar sol-vent mixtures do not mix well with hydrophilic luminol reagents in a post-column reaction coil.

Development of a fl w injection analysis (FIA) system coupled with a fluorescenc de-tection system enables determination of hydroperoxides in fis muscle at picomole levels during the early stages of lipid oxidation. The FIA system is employed for quantitative determination of total lipid hydroperoxides in commercially available edible oils, including vegetable oils and fis oils. The FIA system can be used not only as a practical alternative to the iodometric titration method but also more sensitive and specifi methodology for quantitative determination of trace amounts of lipid hydroperoxide. Especially in evaluating
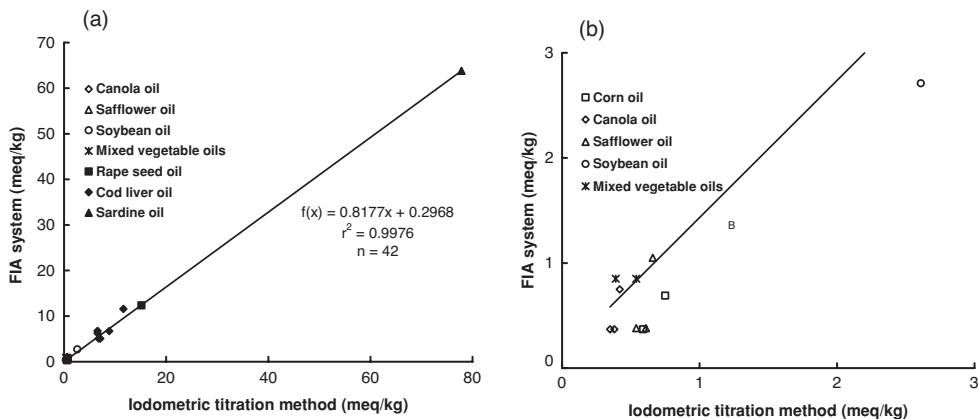
Fig. 8.1   Relationship between the total lipid hydroperoxide contents determined by the FIA system and that by the official AOCS iodometric titration method Cd 8-53 (1990). (a) PV between 0 and 80 meq/kg by iodometric titration and (b) PV between 0 and 3.2 meq/kg by iodometric titration. Adapted from Sohn et al. [26], with permission of AOCS Press.

the early stage of oxidation of lean fis   muscles, the FIA system shows sufficien sensitivity and reproducibility (Fig. 8.1) [26].

8.3   Lipid oxidation in ordinary and dark muscle of fish

It is important to monitor the progress of lipid oxidation in fis   fles   at the early stages when evaluating the development of rancid off-odour in raw fis   fles   due to lipid oxidation. There have been few systematic studies that elucidate the relationship between lipid peroxidation and rancid off-odour development in fresh fis   fles   [27].

The predominant constituent fatty acids of ordinary and dark muscles are 16:0, 16:1 n-7, 18:1 n-9, 20:5 n-3, and 22:6 n-3 (Table 8.1). The content of PUFA is markedly higher in dark muscle compared to ordinary muscle among all fis   species [28].

The total lipid hydroperoxide content in the yellowtail ordinary muscle is generally low and usually remains unchanged during ice storage. On the other hand, total lipid hydroperoxide content in the dark muscle is significantl   higher than ordinary muscle throughout the storage time, even though the initial amount in the dark muscle is close to that of the ordinary muscle prior to storage. The total lipid hydroperoxide content in the ordinary muscles of certain fis   increased at a slower rate than in the dark muscles. For Pacifi saury, Japanese Spanish mackerel, and chub mackerel, both dark and ordinary muscles tended to show an increase in hydroperoxide. However, the total lipid hydroperoxide content in the ordinary muscle of chub mackerel increased rapidly and exceeded that in the dark muscle after ice storage (Fig. 8.2) [28]. There was no correlation between the ratio of lipid content and total lipid hydroperoxide between ordinary and dark muscles of fis   (Table 8.2) [28]. Thus, total lipid hydroperoxide accumulation in the early stage of lipid oxidation differed not only between fis   species but also between ordinary and dark muscles.

No significan correlation between the intensities of smell and total lipid hydroperoxide content existed in the ordinary muscle. However, there was a significan correlation between the intensity of rancid off-odour and total lipid hydroperoxide content in the dark muscle (Fig. 8.3) [29]. A significan relationship existed between the intensity of overall smell and

Table 8.1 Fatty acid compositions (%) of ordinary and dark muscles of various fish species. Adapted from Sohn et al. [28], with permission of Blackwell Publishing Ltd.

Fatty acid	Yellowtail		Amberjack		Japanese butterfish		Pacific saury		Japanese Spanish mackerel		Chub mackerel	
	Ordinary	Dark	Ordinary	Dark	Ordinary	Dark	Ordinary	Dark	Ordinary	Dark	Ordinary	Dark
14:0	44.4	8.38	4.56	11.16	1.25	3.39	0.99	11.41	2.12	3.12	2.05	2.68
15:0	1.09	1.68	0.41	1.25	0.39	0.93	0.10	1.14	0.21	0.30	0.52	0.78
16:0	20.93	35.74	9.94	38.35	8.66	23.40	2.57	24.66	11.68	16.82	13.42	19.36
17:0	0.77	1.16	nd	nd	0.42	1.05	0.08	0.80	0.22	0.32	0.57	0.97
18:0	4.29	8.03	2.00	10.79	3.24	8.11	0.53	4.03	2.50	3.62	3.31	5.08
Saturated	31.52	54.99	16.91	61.55	13.96	36.88	4.27	42.04	16.73	24.18	19.87	28.87
16:1 n-7	6.73	10.57	4.72	15.40	2.15	5.86	0.47	5.33	3.67	5.64	2.28	3.39
18:1 n-9	16.22	30.51	8.07	46.49	13.61	35.52	0.78	8.08	13.32	17.71	10.81	16.11
18:1 n-7	2.71	5.15	1.43	nd	1.55	4.00	0.18	2.10	2.18	3.23	1.60	nd
20:1 n-9	nd	nd	nd	nd	1.49	3.80	1.90	24.03	0.18	1.14	1.04	1.69
20:1 n-7	1.19	2.51	1.39	8.60	0.39	1.12	0.59	6.33	0.13	0.19	0.27	0.43
22:1 n-11	0.35	0.29	0.74	6.27	0.34	1.13	3.05	35.99	0.53	0.92	0.21	0.42
22:1 n-9	0.95	1.23	nd	nd	0.60	1.98	0.17	1.76	0.15	0.24	0.46	0.82
Monoenoic	28.15	50.26	16.35	76.76	20.13	53.41	7.14	83.62	20.16	29.07	16.67	22.86
16:2 n-4	0.89	1.56	0.23	1.18	0.37	0.86	nd	nd	0.29	0.42	0.25	0.67
16:3 n-4	nd	nd	0.54	0.93	0.17	0.45	nd	nd	0.28	0.48	0.29	0.48
18:2 n-6	2.42	3.61	2.08	8.38	0.21	0.60	0.25	2.69	0.47	0.72	0.53	0.81
18:3 n-3	1.01	1.54	0.54	1.93	0.12	0.34	0.21	1.94	0.25	0.40	0.36	0.41
18:4 n-3	1.08	1.54	0.76	2.36	nd	0.23	0.68	5.64	0.51	0.76	0.40	0.43
20:4 n-6	1.78	2.55	0.62	2.60	1.32	3.17	0.10	0.95	0.55	0.79	1.07	1.64
20:4 n-3	0.51	0.74	0.34	1.41	0.16	0.44	0.18	1.71	0.22	0.33	0.23	0.28
20:5 n-3	6.09	9.02	4.44	16.25	1.70	4.60	1.07	8.88	4.24	6.10	3.03	3.90
22:3 n-6	0.31	0.63	0.26	0.61	nd	nd	0.07	0.54	0.19	0.28	nd	0.16
22:5 n-6	1.00	1.59	nd	nd	0.70	1.75	nd	0.49	0.20	0.35	0.58	1.04
22:5 n-3	2.36	3.71	1.08	5.67	0.91	2.57	0.26	3.11	0.91	1.47	0.74	1.17
22:6 n-3	18.19	25.77	7.91	40.23	3.60	10.26	3.55	26.91	8.19	13.82	8.95	15.19
Polyenoic	35.64	52.26	18.80	81.55	9.26	25.27	6.37	52.86	16.30	25.92	16.43	26.18
Total	95.31	157.51	52.06	219.86	43.35	115.56	17.78	178.52	53.19	79.17	52.97	77.91

Abbreviation: nd, not detected.
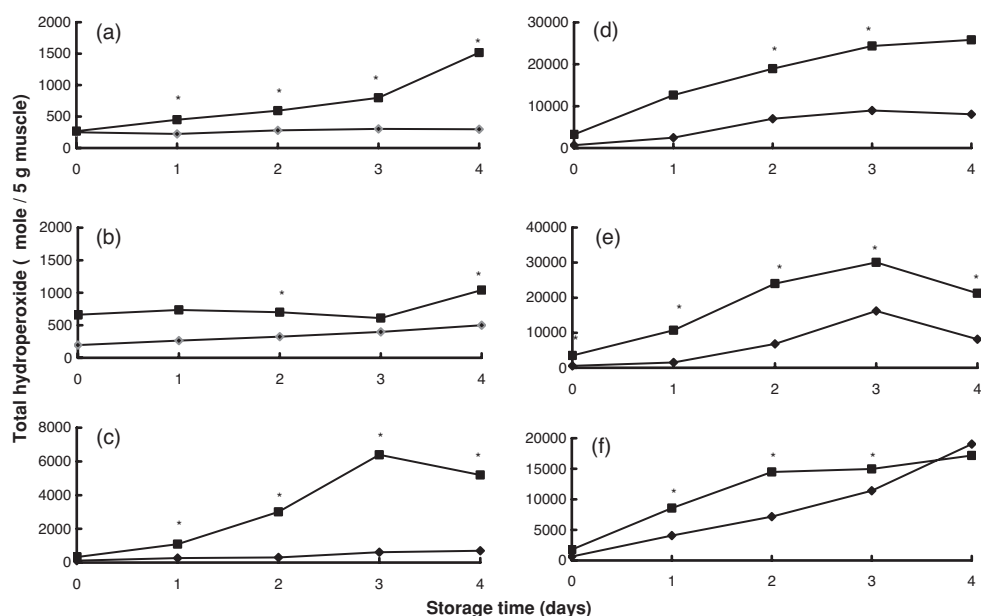
Fig. 8.2   Changes in total lipid hydroperoxide in ordinary and dark muscles of fishes during ice storage for 4 days. (a) Yellowtail; (b) Amberjack; (c) Japanese butterfish; (d) Pacific saury; (e) Japanese Spanish mackerel; and (f) Chub mackerel. Significant differences ($P < 0.01$) between ordinary (□) and dark (■) muscles are represented with an asterisk (*). Arrows represents the first recognition of rancid off-flavour. Adapted from Sohn *et al*. [28], with permission of Blackwell Publishing Ltd.

Table 8.2   Lipid content and total lipid hydroperoxide for ordinary and dark muscles of various fish species. Adapted from Sohn *et al*. [28], with permission of Blackwell Publishing Ltd.

Fish	Lipid contents (g/100 g muscles)		Total lipid hydroperoxides (μmol/g lipid)	
	Day 0	**Day 4**	**Day 0**	**Day 4**
Yellowtail				
Ordinary muscle	8.0	7.4	0.63	0.80
Dark muscle	16.2	12.2	0.33	2.49
Amberjack				
Ordinary muscle	3.4	4.1	1.14	2.43
Dark muscle	15.2	14.3	0.87	1.56
Japanese butterfish				
Ordinary muscle	5.6	3.7	0.41	3.79
Dark muscle	12.6	11.2	0.52	9.26
Pacific saury				
Ordinary muscle	2.7	2.5	4.96	64.61
Dark muscle	24.8	23.5	2.62	21.98
Japanese Spanish mackerel				
Ordinary muscle	6.6	7.1	1.65	22.97
Dark muscle	11.0	12.8	6.39	33.16
Chub mackerel				
Ordinary muscle	8.0	6.7	1.61	56.97
Dark muscle	8.5	8.8	4.20	39.02
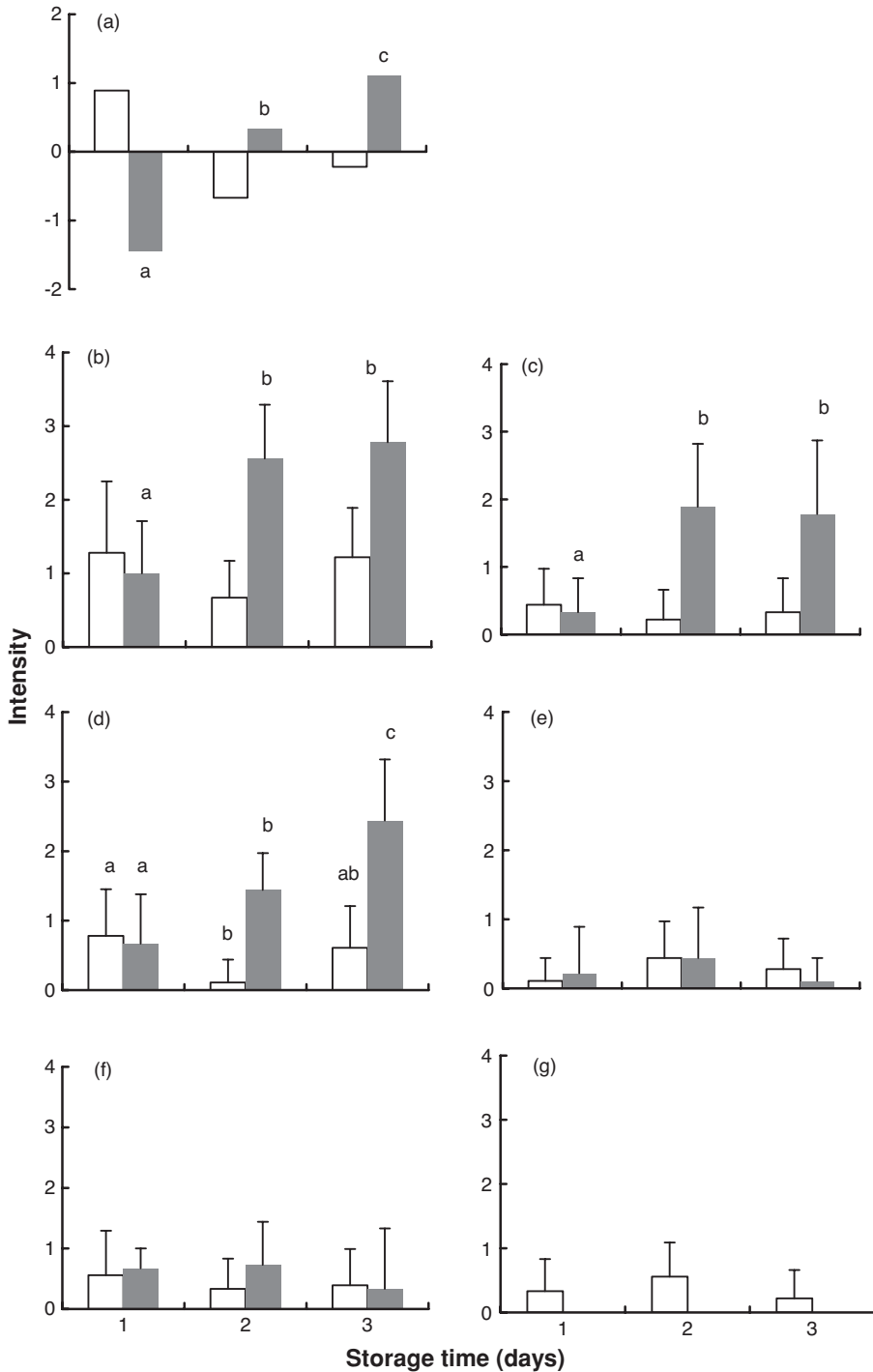
Fig. 8.3 Changes in odour intensities in ordinary and dark muscles of yellowtail during ice storage for 2 days. (a) Overall smell; (b) Fishy smell; (c) Spoiled smell; (d) Rancid off-odour; (e) Grassy smell; (f) Bloody smell; and (g) Milky smell. For each muscle at the same storage periods, mean values with different superscripts (a–c) are significantly different ($P < 0.05$). (□), ordinary muscle and (■), dark muscle. Adapted from Sohn *et al.* [28], with permission of Blackwell Publishing Ltd.

total lipid hydroperoxide content. Both the accumulating rates and the actual amount of total lipid hydroperoxide in the ordinary muscles of fis vary greatly from species to species [28].

The most important indices by which consumers evaluate the freshness and quality of raw muscle foods are colour and fl vour [30]. Haemoglobin and myoglobin are the most abundant haem proteins in blood and dark muscle, respectively. Moreover, the differences in the colour tone of ordinary muscles among fis species are mainly due to different ratios of oxymyoglobin and metmyoglobin. The bright red colour of oxymyoglobin changes to brown or dark-brown because of the oxidation of myoglobin. Myoglobin is the predominant pigment in most fis muscles and it is well-known that the high myoglobin content in dark muscles contributes to the reddish-brown colour of the flesh The rate of muscle discoloration is closely related to the rate of oxymyoglobin oxidation [31,32]. The contribution of haem protein to lipid peroxidation has been suggested [33–37] and it has been reported that the lipid oxidation in fis muscle was promoted by autoxidation of myoglobin, suggesting a close relationship between lipid oxidation and myoglobin oxidation. Moreover, O'Grady *et al*. [38] reported a relationship between oxymyoglobin oxidation and lipid oxidation in bovine muscle. It is believed that the formation of metmyoglobin by the oxidation of myoglobin predominantly in dark muscle accelerates lipid oxidation and leads to the generation of greater amounts of hydroperoxides. Thus, lipid oxidation associated with metmyoglobin formation may cause the development of a rancid off-odour and fis y smell in the dark muscle. The ordinary muscle of yellowtail, amberjack, and other white muscle fish contain a low level of metmyoglobin and the influenc of myoglobin oxidation on the development of a rancid off-odour appears to be insignificant On the other hand, the ordinary muscles of chub mackerel and skipjack contain high levels of hydroperoxide and the decomposition of this hydroperoxide may contribute to the development of a rancid off-odour.

8.4   Effects of bleeding and perfusion of yellowtail on post-mortem lipid oxidation of ordinary and dark muscles

Haemoglobin and myoglobin are the predominant haem proteins in red blood cells and the dark muscle tissues of a variety of animals, respectively. The colour tone of fis muscles depends on the ratio of myoglobin and haemoglobin contents in the muscles [39,40]. Moreover, methaemoglobin and metmyoglobin, the oxidized analogues of haemoglobin and myoglobin, respectively, usually contribute to lipid oxidation in fis fles [33,34,41]. Haem compounds are among the main components that catalyze lipid oxidation of fresh meat. Contrary to this, it has also been reported that metmyoglobin has little or no prooxidant activity in cooked beef meat [42].

Generally, bleeding of fis is carried out to eliminate most of the haemoglobin from the tissues. Haemoglobin is highly concentrated in the erythrocyte and haem iron is a major catalyst of lipid oxidation in fis flesh It is well accepted that immediate bleeding of fis results in the delay of rigor mortis compared to unbled fles during ice storage. On the other hand, residual blood in the fis tissues is one of the main factors that lead to the development of undesirable discoloration [43] and unpleasant fl vour during ice storage. Therefore, fis without bleeding treatment is usually unsuitable for "sashimi" and "sushi" because of undesirable colour and odour. The rate of progress of rigor mortis in horse mackerel muscle with bleeding was slower than the muscle without bleeding [44]. Immediate bleeding

of skipjack showed that the bled muscle maintained a bright colour and fresh smell compared to the unbled flesh due to the formation of a lesser amount of metmyoglobin and higher pH [45]. Bleeding treatment causes the delay of muscle softening in pelagic species such as yellowtail, horse mackerel, and striped jack but not in demersal species such as red sea bream, flatfis and rudder-fis [46]. However, little information is available on the effects of bleeding on lipid oxidation of fis fles [47,48].

Total lipid hydroperoxide contents of the bled compared to unbled yellowtail ordinary muscles showed significan difference during ice storage. The total lipid hydroperoxide contents of bled ordinary muscle were higher than unbled ordinary muscle throughout ice storage. On the other hand, the total lipid hydroperoxide contents of dark muscles were higher and increased more rapidly compared to those of ordinary muscles. Total lipid hydroperoxide contents of unbled dark muscle were significantl higher than that of bled dark muscles [49].

There is no significan difference in the metmyoglobin percentage between bled and unbled muscles. On the other hand, metmyoglobin levels in the dark muscles were higher and proceeded rapidly compared to that of ordinary muscles and it accumulated more than two-fold of metmyoglobin percentage than that in ordinary muscle (Fig. 8.4) [29].

Bleeding of fis aims to remove undesirable effects of blood on fis muscle quality, including fl vour, texture, consistency, and appearance [45,46]. Several authors have demonstrated the effect of bleeding on muscle in terms of lipid peroxidation. The important reaction of haemoglobin to stimulate lipid peroxidation is mainly due to the oxidation of haemoglobin. The bleeding process is carried out to remove all the blood from the fis body. Thus, bleeding prevents catalytic action of haemoglobin in lipid oxidation in fis muscle. On the other hand, Porter *et al*. [47] reported that there was no significan difference in lipid oxidation between
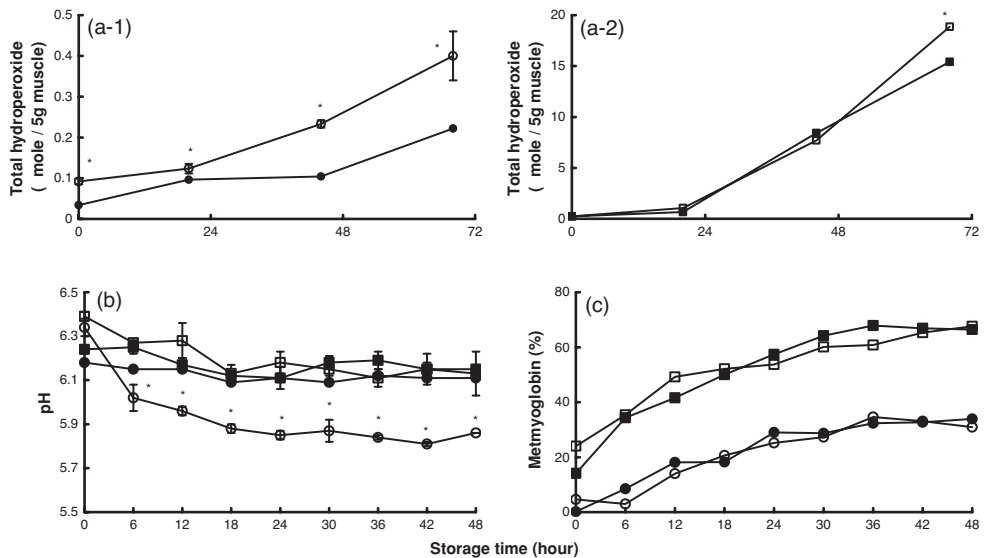
Fig. 8.4 Changes in total lipid hydroperoxide (a), pH (b), and percentage of metmyoglobin (c) of yellowtail muscle during ice storage. (a-1) Ordinary muscle and (a-2) Dark muscle. For muscle, significant differences ($P < 0.01$) between muscles with and without bleeding treatment are indicated by asterisks. ○, Ordinary muscle with bleeding treatment; ●, Ordinary muscle without bleeding treatment; □, Dark muscle with bleeding treatment; and ■, Dark muscle without bleeding treatment. Adapted from Sohn *et al*. [29]. Copyright 2007, with permission from Elsevier.

bled and unbled salmon muscle during frozen storage. Sohn *et al*. [29] showed that bleeding of ordinary and dark muscles delayed the formation of total lipid hydroperoxide compared to those of unbled muscles. The difference in the amounts of total lipid hydroperoxide was not confirme between ordinary and dark muscles of fish This difference may be due to the feed, age, and environmental conditions as well as the level of myoglobin in the muscle. On the other hand, the bleeding process did not suppress the accumulation of total lipid hydroperoxides in the ordinary and dark muscles of yellowtail during ice storage (Fig. 8.4). Moreover, total lipid hydroperoxide contents in bled ordinary muscle were higher and increased compared to that in unbled ordinary muscle. Itazawa *et al*. [48] reported that whole content of blood in yellowtail body was 48.9 ± 10.7 mL/kg. Bleeding of yellowtail removed the blood of 12.8 ± 1.2 mL/kg, which is equivalent to $49.6 \pm 6.0\%$ of blood cells. The haemoglobin and myoglobin contents in ordinary and dark muscles of yellowtail were 30 and 560 to 800 mg/100 g muscle, respectively [46]. Most of the haem proteins in ordinary muscle were myoglobin [39]. Thus, bleeding treatment did not reduce the concentration of haemoglobin sufficientl to reduce lipid oxidation, although removal of the blood from the muscle resulted in the delay of muscle softening. It is confirme that the bleeding did not influenc lipid oxidation of yellowtail muscle by comparing the perfused muscle where blood was removed by perfusion. The total lipid hydroperoxide content in dark muscles were much higher and rapidly increased compared to that in the ordinary muscle. The high content of haemoglobin and myoglobin in the dark muscle might explain the greater extent of lipid oxidation in dark muscle as compared to that in ordinary muscle, suggesting that lipid oxidation of yellowtail whole muscle was predominantly due to the dark muscle protein.

Lactic acid generated in anoxic conditions from glycogen is a principal factor in lowering the post-mortem pH in fis muscles [49]. The ordinary muscle with bleeding treatment may be in an anoxic condition during treatment. The dark muscles with and without bleeding treatments are slightly higher in pH than that in ordinary muscles. However, the pH of the dark muscles showed no significan difference in the muscles with and without bleeding treatments.

Ascorbic acid is commonly used to prevent undesirable oxidative changes in fles meat or various foodstuffs. Indeed, addition of ascorbic acid prevents lipid oxidation and colour changes in ground beef [50]. On the other hand, ascorbic acid sometimes acts as a prooxidant in foods. Ohshima *et al*. [37] reported that the addition of ascorbic acid accelerated lipid oxidation in cooked mackerel meat during storage at $4°C$. This prooxidant effect of ascorbic acid was also observed in the homogenate of the Japanese oyster [51]. Yin *et al*. [52] reported that the prooxidant effect was enhanced more strongly by increasing the concentration of ascorbic acid. It acted as a prooxidant at lower concentrations, but as an antioxidant at higher concentrations when added to meat [53]. The function of ascorbic acid added to the fles is affected by many factors, including unsaturated fatty acids, enzymes, metal ions, and storage conditions. The addition of ascorbic acid to dark muscle of yellowtail by the perfusion treatment did not show any effects on delaying the accumulation of total lipid hydroperoxide. The oxidation reaction of ascorbic acid probably occurred in the dark muscle of yellowtail. Therefore, the radical species generated from oxidized ascorbic acid may act as a prooxidant, and it might accelerate the accumulation of total lipid hydroperoxide in the dark muscle.

The effect of hydrophilic Trolox®, a hydrophilic analogue of tocopherol in which a carboxylic-acid group is replaced by a phytol side-chain of tocopherol [54], in preventing lipid oxidation in muscles has been examined. The effects of Trolox® against oxidative damages, particularly against lipid and myoglobin oxidation, have been reported in animal muscle [38,55,56]. Antioxidative effects of Trolox® compared to ascorbic acid group have

been reported [29]. Thin layer tests in vegetable oils and animal fats have shown that Trolox[®] had two to four times higher antioxidant activity than butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), and was more active in terms of antioxidative properties than propyl gallate, ascorbyl palmitate, and α- and γ-tocopherols [54]. Trolox[®] delayed metmyoglobin formation in yellowtail muscle compared to the control groups [29]. These results suggest that the Trolox[®] acts as a scavenger of free radicals under hydrophilic conditions of fis muscles during the early stages of lipid oxidation.

The modern FIA system accomplished quantitative determination of total hydroperoxides with high reproducibility, even at extremely low concentrations of hydroperoxides. Moreover, it is possible to estimate lipid oxidation not only in lean fish but also in minor fis muscle parts, such as the dark muscle. Well controlled oxidation of fis lipids will maintain acceptable freshness and nutrition characteristics of fis and will expand possibilities of fis products for processing and during storage.

8.5   Conclusions

It has been accepted that progress of lipid oxidation in fis muscles varies, not only among fis species but also between muscle types. Most of the data were collected by using traditional evaluation methods for lipid oxidation, such as thiobarbituric acid-reactive substances (TBARS). Secondary oxidation products of lipids usually possess characteristic off-odours that are usually undesirable. Therefore, comparison of lipid oxidation patterns among fis species at the very early stage of storage is one of the important issues in the understanding of lipid oxidation. In this chapter, a fl w injection analysis system for the measurement of hydroperoxides was introduced as an effective tool to evaluate lipid oxidation in raw fis muscle during the early stages of storage. The proposed system accomplished quantitative determination of total hydroperoxides with high reproducibility, even at extremely low concentrations. Moreover, it was possible to estimate lipid oxidation not only for low fat fish but also for small part of fis muscle such as dark muscle.

Lipid hydroperoxides accumulation in fis muscles during early stages of lipid oxidation differs among fis species as well as between the ordinary and the dark muscles. On the other hand, lipid oxidation in both ordinary and dark muscles of yellowtail contributes to the development of a rancid off-odour in the early stage of ice storage. The high content of haemoglobin and myoglobin in the dark muscle might explain the greater extent of lipid oxidation in the dark muscle when compared to that in the ordinary muscle. Lipid oxidation of fis muscle might predominantly be due to the dark muscle protein. The removal of residual blood alone from yellowtail did not delay lipid oxidation in the early stage of ice storage. To prevent lipid oxidation in the yellowtail dark muscle, the addition of an antioxidant such as Trolox[®] may be necessary. Well controlled oxidation of fis lipids will maintain acceptable freshness and nutritional integrity of fis and will expand possibilities of fis products processing and storage.

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9 Blackening of crustaceans during storage: mechanism and prevention

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9.1 Introduction

Food products, including those from fisherie resources, have three critical characteristics: nutritional, palatable, and bio-modulating properties. The second property involves taste, aroma, texture, and appearance, all of which are tightly linked to the attractiveness of foods for consumers. In particular, food colour is an influentia determinant, which is derived from puteridines, indoles, chlorophylls, carotenoids, fl vonoids, ommochromes, tetrapyroles and other pigments. Among pigments, some elevate the commercial value of seafood products while others suppress it. For example, the bright body colours of fish such as red sea bream, yellow tail, and rainbow trout, which are mainly due to some xanthophylls, fascinate consumers. In contrast, black pigments such as melanin severely damage the market value of certain seafood.

Prawns are a very important fisher resource all over the world because of their high market value (Table 9.1). Prawns are usually frozen during transportation and storage, but they are sensitive to discoloration after thawing, becoming black on their heads and tails. Such discoloration severely reduces their market value.

Melanin is present in species ranging from bacteria to humans [2]. While much is now understood about the systems governing melanogenesis and the nature of melanins synthesised in mammals, little is known about melanins produced by invertebrates. Melanins produced by different species look similar, but there are many differences in melanogenesis between vertebrates and invertebrates, including prawns.

While in vertebrates, melanin is produced by the enzyme tyrosinase in melanocytes, melanogenesis in prawns occurs in the hemolymph and in the cuticle, and results from enzymatic phenol-oxidation catalyzed by a so-called "phenol oxidase" (PO). The key reactions of PO are to catalyze the conversion of mono- and diphenols into *o*-quinones, which leads to melanin formation [3–5].

In arthropods, including crustaceans, PO is expressed in hemocytes as an inactive precursor (proPO) and functions in various phases of the living body such as sclerotization, pigmentation, wound healing in the cuticle and defence reactions. In the case of immune responses, the activation of proPO to PO is regulated by a serine protease called the proPO activating enzyme, which is triggered by cell wall components (lipopolysaccharides,

Table 9.1   World trade of shrimps and prawns, Adapted from FAO, 2008 [1]

		1980	1985	1990	1995	2000	2005	2006
Import	Value	2,295,359	3,117,838	6,348,323	9,053,190	9,657,588	9,518,349	10,423,409
	Quantity	361,862	524,093	903,656	998,904	1,218,622	1,568,377	1,654,080
Export	Value	2,032,156	2,738,455	5,895,754	8,287,531	8,329,902	8,972,506	9,540,144
	Quantity	338,768	485,283	869,273	961,382	1,120,881	1,636,569	1,596,655

Value: US$1000.
Quantity: metric tons.

peptidoglycans or beta-1,3-glucans) of bacteria or fungi [3–5]. It has been widely accepted that the post-harvest conversion of proPO to PO presumably occurs in a manner similar to that observed in the living body and may induce melanogenesis during storage, although there remains much to be investigated.

However, understanding the functions of PO has been mainly in insects, which are classifie into a different class (Insecta) and prawns (Crustacea), although they belong to the same phylum. Indeed, they share many physiological characteristics, but several properties clearly differ; for example, many insects do not contain oxygen transporter molecules owing to their developed trachea, while prawns depend on the respiratory pigment hemocyanin (Hc); insects excrete nitrogen as uric acid in Malpighian tubules, while prawns excrete it as ammonia in antennal glands; insects harden their cuticles by cross-linking proteins, while prawns do so by deposition of calcium. Little investigation on the phylogenetic relationship of phenol-oxidation has been performed in arthropods, although there are two other classes in that Phylum – Chelicerate and Polychaeta. Therefore, despite these discrepancies, find ings about the mechanism(s) of insect melanogenesis have been applied to understand the post-mortem blackening of crustaceans.

There have been several reports about melanogenesis in prawns caused by PO. However, the reports have mainly focused on the crude enzyme from whole heads and cuticle, or otherwise on enzyme purifie from the cuticle. No data have been reported about the direct interrelationship of post-harvest blackening and PO from hemocytes. It is well-known that PO and its precursor are very unstable proteins, which easily aggregate and are inactivated, even in the course of experimental procedures. Melanogenesis of prawns, on the other hand, occurs after shipment and storage, a process that lasts for several months. Thus, we have only an incomplete basis to explain this phenomenon. However, following breakthroughs in molecular biology and biochemical techniques, the oxygen transporter Hc, which is highly homologous with proPO in its copper binding domain, has turned out to be a potent inducer of prawn melanosis, being converted into a highly stable PO-like enzyme during the freezing and thawing process. In this chapter, we summarise recent finding of melanogenesis and its related factors in order to investigate how best to effectively prevent this problem.

9.2   Phylogenetic position of prawns: the relation of PO and Hc

Prawns are classifie as "crustaceans" in arthropods, which mainly includes the four classes of crustaceans, insects, chelicerates and myriapods. Arthropods account for more than 80% of described living animal species, which are characterised by the possession of a segmented

Table 9.2   Presence of hemocyanin (Hc) and proPO in arthropods

	Animals	proPO	He
Crustacean	Prawn, crab	Presence	Presence
Insect	Silkworm, mosquito, fruit fly	Presence	Absence
Chelicerate	Horseshoe crab, spider	Absence	Presence
Myriapoda	Centipede	Presence	Presence

body with appendages on each segment. All arthropods are covered by a hard exoskeleton made of chitin, a polysaccharide, which provides physical protection and resistance to desiccation. Periodically, arthropods shed their covering when they moult. Table 9.2 shows the presence of proPO and Hc in arthropods. Crustaceans, myriapods and insects contain proPO, which is from hemocytes, while there has been no report about proPO in chelicerates. In contrast, insects generally do not have Hc because they have developed a tracheal system that directly transports oxygen into their tissues. The key point here is that crustaceans, such as prawns, express both proPO and Hc.

9.3   Biosynthetic pathway of melanin

Melanin is, no doubt, a key molecule that induces the blackening of prawns; however, research focusing on melanin itself is surprisingly scarce, even in popular experimental animals such as mice or humans. There have been no reports about the chemical characterisation of melanin itself in arthropods, including insects and crustaceans. Since melanin is insoluble in water, this leads to difficultie in its chemical analysis and, unlike other biopolymers such as nucleic acids, proteins, fatty acids and glycochains, melanin does not have a determined chemical structure. However, using cell biological and biochemical techniques, it has been clarifie that mammals and arthropods have distinct melanin synthetic pathways, although they share some key reactions. Here we describe the biosynthetic pathway of melanin in arthropods, mainly from insects (of which the most knowledge has been accumulated) in comparison with that in humans.

Figure 9.1 shows the biosynthetic pathway of melanin in insects [5]. Dopachrome, a reddish-brown intermediate, can be produced from both tyrosine and dihydroxyphenylalanine (DOPA) in insects. However, the reaction mechanism is still controversial. One possibility is that PO directly attacks both tyrosine and DOPA to produce dopaquinone. That intermediate is further converted into 5,6-dihydroxyindole (DHI), which is oxidised by PO to polymerise into black eumelanin. The reaction catalyzed by PO can be substituted by Hc-derived phenoloxidase. Pheomelanin, a light-coloured melanin, which contains sulphur due to its cysteine content, has not been identifie in arthropods. Physiologically, melanisation plays a key role in biodefence, cuticle hardening and wound healing in arthropods. The pathway in mammals is well summarised by Ito and Wakamatsu [6].

9.4   Significance of melanisation in arthropods: pre-harvest and post-harvest

The phenol oxidation system in prawns is shown in Fig. 9.2a. This leads to an understanding of the pre- and post-mortem melanisation of prawns. In a living body, this system is strictly
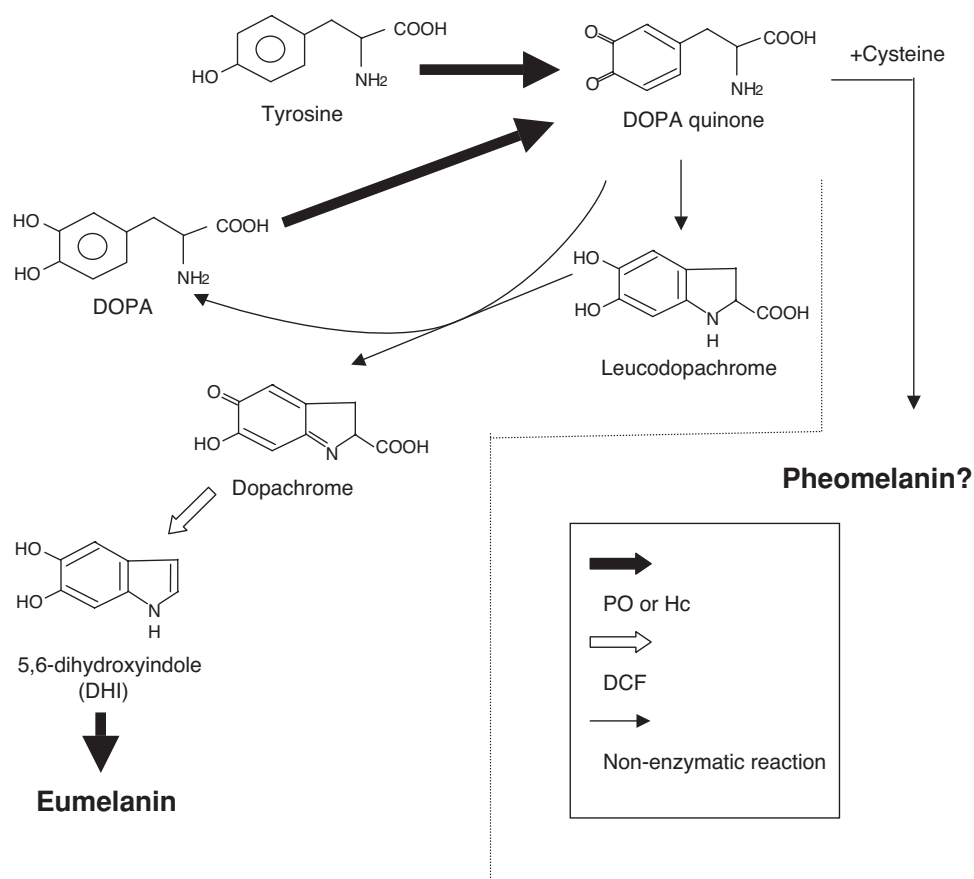
Fig. 9.1 Melanin synthetic pathway in arthropods.

regulated by homeostasis because derivatives of phenol oxidation, such as quinone, are highly toxic to the body as well as to invaders. However, the invasion of pathogens, moulting and wounds stimulates this system to oxidise phenolic compounds, such as tyrosine, in order to produce melanin. In the course of the oxidation process, the radicals or quinone derivatives produced play a crucial role in biodefence and wound healing. However, the level of melanin produced in the living body is very low and does not affect their appearance. This system is strictly regulated in the living body; however, once they are sacrificed that balance breaks down to stimulate the system. As a result, black melanin is produced to induce blackening of prawns.

9.5 Biochemical characterisation of proPO and PO

Phenol oxidase generally exists as an inactive precursor termed proPO. It has long been believed that PO is the only factor catalyzing the phenol-oxidation in prawns, although there has been some indication that proPO is a very unstable protein that becomes sticky and easily aggregates upon simultaneous activation, which makes its purificatio and characterisation

(a) Phenol oxidation in prawns   **(b) proPO System and proPO activation**
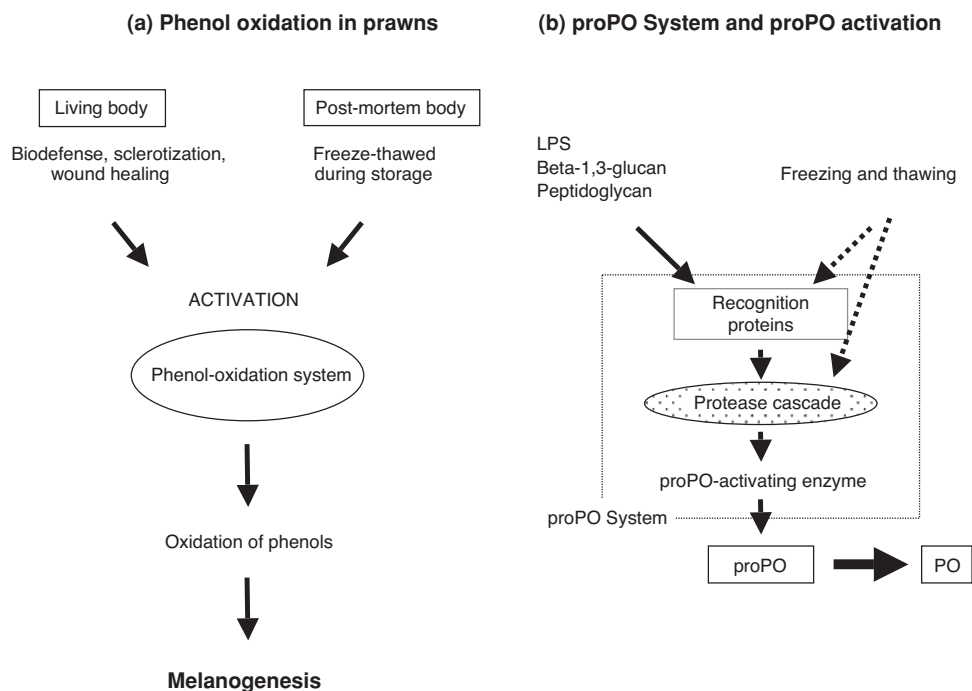
Fig. 9.2 (a) Phenol oxidation in prawns. (b) proPO System and proPO activation: In the case of biodefense, the recognition of beta-1,3-glucans, lipopolysaccharide (LPS) and peptidoglycans by specific proteins triggers the protease cascade, which leads to limited cleavage of proPO that converts it to PO. The molecules involved in this system are called the proPO System. The effects of the freeze-thawing process on this system remain unclear, but it is believed that this system is strictly controlled in the living body and breaks down post-mortem to spontaneously activate proPO to PO.

difficul [7–9]. The details of characterisation of proPO are described by Cerenius and Söderhäll [3].

9.6   The relationship of PO and melanogenesis in prawns

It has generally been thought that the strictly regulated "proPO System" loses its balance in post-mortem and activates proPO leading to melanin formation (Fig. 9.2b). In 1995 and 1996, two reports were published that led to novel finding concerning the mechanism of blackening. The firs showed the molecular phylogenetic position of proPO using a molecular biological technique that indicated that proPO in crayfis   has a very close relationship to Hc, a well-known oxygen transporter in crustaceans [3]. The second showed conversely that Hc in crabs and lobsters has *o*-diphenol oxidase activity when treated with perchlorate [10]. In 2001, based on those two facts, prawn Hc was shown to be converted into a PO-like enzyme by dodecyl sodium sulphate (SDS) [9]. In addition, this catalytic activity is very stable and is maintained over two months under frozen conditions, while PO loses its activity within a week under the same conditions (Fig. 9.3a). However, the experiment was conducted under *in vitro* conditions because Hc was activated by SDS, which is not an endogenous factor of prawns. In 2003, Hc was reported to be activated by hemocyte component(s), presumably
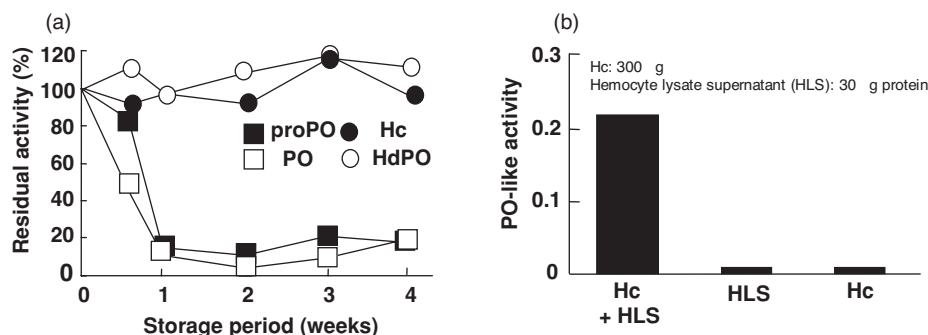
Fig. 9.3 (a) Stability of proPO and Hc under frozen conditions: The residual activity of purified proPO and Hc both in active (□: PO, ○: Activated Hc) and in inactive (■: proPO, ●: Hc) forms. The proteins were kept at −25°C and the phenol oxidative activities were periodically measured. Adapted with permission from Adachi *et al.* [9]. (b) Enzymatic conversion of Hc into a PO-like enzyme by hemocyte lysate supernatant. PO activity was measured after the addition of a hemocyte lysate supernatant (HLS) to purified Hc. The reaction mixture contains p-APMSF to inhibit proPO activation in hemocytes and beta-1,3-glucan (lanimarin) to elicit the activation factor. Adapted from Adachi *et al.* [11]. Copyright 2003, with permission of Elsevier.

by serine/cysteine proteases, which are triggered by beta-1,3 glucans like the proPO system [11] (Fig. 9.3b). This fact has two important implications, since it means that Hc can be stimulated by an endogenous factor and that it also plays a crucial role in the biodefence of crustaceans as well as a respiratory pigment. Activated Hc can catalyze three reactions in the melanogenic pathway. Moreover, Hc is a highly concentrated protein, which comprises 90% of hemolymph proteins, estimated to be several mg per prawn, which is about one thousand times higher than that of proPO. These lines of evidence strongly indicate that the true problem in blackening might be Hc rather than PO.

9.7 Hemocyanin and its enzymatic activation

Hc was firs identifie as an oxygen transporter, which is composed of about 650 amino acid residues to form a 72 kDa protein [12,13]. Hc as well as proPO contains two copper binding domains, but Hc differs from proPO in the following three aspects:

1) Hc is modifie by a glycochain;
2) Hc has a signal peptide that is indispensable for its secretion from the cell;
3) Hc does not contain a thiol ester region.

While proPO becomes sticky in the active state, no report about stickiness in Hc has been published. Hc is present in hemolymph at quite high concentrations (more than several mg/mL hemolymph) while the concentration of PO is very low (less than several μg/mL hemolymph). The specifi activity of PO for DOPA oxidation is 5- to 10-fold higher than that of Hc [9]. Recently, the presence of Hc in the exoskeleton has been identified which coincides with the fact that blackening proceeds in their cuticle as well as in the hemolymph. Hc is actually a multifunctional protein that works as a storage protein in moulting [14], as an osmolyte [14], as an ecdysone transporter [15] and as a precursor of an anti-fungal

Table 9.3 Biochemical comparison of proPO and Hc

	Tissue expressed	Location	Function	Stability	Concentration
proPO	Hemocyte	Hemocyte Serum Cuticle	Phenol-oxidation	Low (inactivated within a week at $-25°C$)	Several µg/mL hemolymph
Hc	Hepato-pancreas	Serum Cuticle	Phenol-oxidation Osmolyte Storage protein Ecdysone transporter Precursor of antifungal peptide	High (active after two months storage at $-25°C$)	Several mg/mL hemolymph

	Specific activity	Domains	Signal peptide	Post-transcriptional modification	Others
proPO	80.6 U	Copper binding domain Thiol ester domain	Non (with exceptions)	Cuticular proPO contains oxidised methionine	Become stick after inactivation
Hc	18.4 U	Copper binding domain	Exist	Glycosylation	

peptide [13]. Hc is activated into a PO-like enzyme by SDS, trypsin, isopropanol, perchlorate or other agents [13]. SDS is a potent activator, regardless of the species, and it affects the conformation around the active centre, leading to easier entrance of the substrate to the catalytic pocket although the mechanism has not been experimentally verified The specifi activity of Hc is significantl lower than that of PO, and no difference has been shown for biochemical properties (e.g. specificit of inhibitor and substrate, optimum temperature and pH, etc.). The most important biochemical characteristic shared with PO is substrate specificit , indicating that Hc can catalyze the same reactions in the melanogenic pathway (Fig. 9.1). A biochemical comparison of Hc and proPO is summarised in Table 9.3.

There have been three reports about Hc activation by endogenous factors. The firs report is in horseshoe crabs, which are classifie as a chelicerate. In these animals, Hc is stimulated by a coagulation factor called "Factor B" and "clotting enzyme", both being serine proteases [13]. What should be noted here is that this activation is not accompanied by limited proteolysis at the *N*-terminus of the subunit that occurs during proPO activation. The formation of a complex of these coagulation factors and Hc triggers the activation. The second report is also from horseshoe crabs, which shows that an antibacterial peptide called tachiplecin also activates Hc [13]. Tachiplecin contains an amphiphilic structure, which suggests that its activation mechanism is identical to that of SDS. The third report is about prawns showing that Hc is converted into a PO-like enzyme by a hemocyte lysate supernatant stimulated by beta-1,3 glucan and is severely inhibited by serine/cysteine protease inhibitors, which indicates that a biodefence-related protease is involved in this system [11]. In addition, this activation is accompanied by the disappearance of S–S bonds between subunits of Hc, the details of which remain unclear. In crustaceans, the Hc activator has not been identifie and the biochemical characterisation of Hc activated by the endogenous factor has not yet been performed.

There has been no report of proPO and the proPO system in horseshoe crabs. It is believed that coagulation factors play a central role in Hc activation. The coagulation system in horseshoe crabs has been biochemically well investigated for a long time and all factors involved and their interactions have been identifie [16]. This system and the proPO System share some properties:

1) they involve the serine protease cascade;
2) the cascade(s) are triggered by polysaccharides; and
3) they are involved in biodefence.

However, coagulation in crustaceans is induced by transglutaminase, while those in horseshoe crabs are induced by insolubilisation of proteins, indicating that they have distinct coagulation systems [17]. Therefore, the system of Hc activation in horseshoe crabs cannot be directly applied to the activation in prawns. However, the most intriguing and mysterious point of the crustacean phenol-oxidation system is why they contain two different systems – proPO and Hc.

9.8 The relationship of frozen storage and blackening

The proposed scheme of blackening that occurs during frozen storage is summarised in Fig. 9.4. The proPO and Hc show no enzymatic activity in the living body (period a). Both factors are enzymatically activated by the "disruption of homeostasis" in the post-mortem
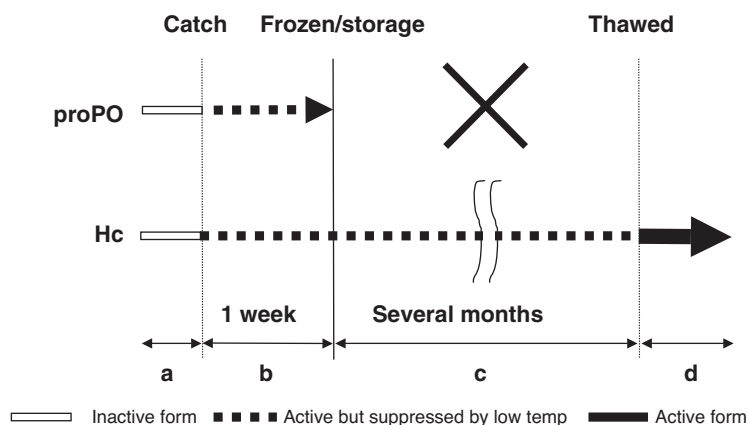
Fig. 9.4 Predicted behaviour of proPO and Hc during frozen storage. From harvesting to consummation, the behaviour of proPO and Hc can be categorized into four stages: (a) Before catch: Both proPO and Hc are in inactive forms, which are strictly regulated in homeostasis of the living body. (b) One week after catch: In the post-mortem stage, both proPO and Hc are activated. However, PO loses its activity under frozen conditions while Hc is converted into an enzymatically active form. (c) Frozen period after the inactivation of proPO: Frozen prawns are normally stored under frozen conditions for several months during distribution, including shipment and storage in warehouses. In this stage, proPO is inactivated and Hc exists in an active form, of which the activity is severely inhibited at low temperature. (d) After thawing: Hc is exposed to above-zero temperatures and begins to oxidize phenols, resulting in blackening.

stage, but their activities are suppressed in frozen conditions (period B). The proPO, an unstable enzyme, is inactivated within a week, even under frozen conditions, while Hc maintains its activity over months (period C). The blackening sometimes proceeds very slowly during the frozen-storage process because the activity is not completely inhibited during that time (period C). Upon shipment, they are thawed and drastic blackening progresses because the enzyme activity of Hc is increased by the higher temperature (period D). It is worth noting that this scheme is deduced from *in vitro* data. In order to clarify the details of blackening in prawns, further investigation is required using various approaches.

9.9   Prevention of melanosis in prawns

Preventive methods for prawn blackening, which are now in use, can be categorised into physical and chemical treatments. Little information is available about blackening focusing on Hc-derived phenyloxidase activity. Therefore, this chapter principally summarised reports about PO-induced blackening. However, considering that their primary structures contain copper, the catalytic mechanisms of PO and Hc seem to be quite similar, suggesting that prevention of PO activity would be applicable to Hc-induced blackening. This topic has been summarised by Kim *et al*. [18], which discusses the potential role of PO in blackening.

9.10   Conclusions

Many papers have reported on the mechanism of the melanin formation of prawns during post-harvest storage. These reports have long discussed the mechanism based on the PO system responsible for biodefence of live crustaceans. In this chapter, it was suggested that Hc is the real culprit for melanin formation, especially in freeze-thawed prawns. As the following stage, the direct involvement of Hc in mediating blackening should be carefully verifie by *in vivo* studies.

Nitrogen or carbon dioxide gas packaging must be useful for preventing blackening, since the deterioration is basically induced by oxygen. Recently packaging materials with properties of high oxygen barrier and anti-pinhole have been developed. The possible application of these novel materials for gas packaging should be considered in the near future.

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10 Quality of freshwater products

Masaki Kaneniwa

10.1 Introduction

Total world fisherie production was about 144 million tonnes in 2006. The major produce
of world fisherie is captured marine species (81.9 million tonnes) while those of inland
aquaculture products, marine aquaculture products, and inland captured products were 31.6,
20.1, and 10.1 million tonnes, respectively [1]. As aquaculture production is increasing year
by year, total fisherie production has also increased gradually. However, there has been little
change in the production of captured fis from marine resources during the past ten years.
On the other hand, inland production has increased and reached about 42 million tonnes
between 1997 and 2006, and now constitutes about 30% of the world fisherie production.
This is mainly due to a 1.8-fold increase in aquaculture products from 1997 to 2006. Major
products of inland aquaculture products are freshwater fish which serve as important protein
resources in the developing countries.

China is the biggest freshwater fis producer, where the common freshwater fis produced
are silver carp, grass carp, common carp, big-head carp, and crucian carp. They are also the
major fis species in world fisherie production (Table 10.1).

In China, most freshwater fis are consumed in large cities, with the freshwater culture
ponds nearby. The production of these freshwater fis will increase in the future by the
development of new aquaculture and processing technologies. The basic nutritional informa-
tion of freshwater fis is necessary for their effective utilisation. Fish species are important
and valuable resources for protein and lipids. Fish lipids can be characterised by their lipid
classes and fatty acid compositions. This chapter focuses mainly on the lipid characteristics
of freshwater fish

10.2 Lipid and fatty acid composition in freshwater fish

The lipid composition of the fis affects the nutritional value and the shelf-life of fis
products. Nutritionists and food scientists require lipid and fatty acid composition data to aid
them in dietary formulation, nutrient labelling, processing, and product development [2].

Table 10.1 Major fish species in world fisheries production in 2006 [36]

Fish species	Production type	Production (million tonnes)
Anchoveta	Marine capture	7.0
Silver carp	Inland aquaculture	4.4
Grass carp	Inland aquaculture	4.0
Common carp	Inland aquaculture	3.2
Alaska pollock	Marine capture	2.9
Skipjack tuna	Marine capture	2.5
Big-head carp	Inland aquaculture	2.4
Atlantic herring	Marine capture	2.2
Crucian carp	Inland aquaculture	2.1
Blue whiting	Marine capture	2.0

The popular freshwater fis species sold in Chinese markets are shown in Table 10.2, along with their lipid characteristics (Table 10.3) [3]. The total lipid (TL) contents of their muscle are 1 to 5% and they are, therefore, categorised as lean (<2%) or semi-fatty (between 2 and 10%) fish such as walleye pollock, red sea bream, or bastard halibut. These Chinese freshwater fis are cultured fish with lipid contents lower than those of cultured marine fis (10–20%). The major components of TL in Chinese freshwater fis muscle are triacylglycerols (TAG) and polar lipids (PL) [3]. Variations of PL contents among fis species are less than those of neutral lipid (NL) contents. Therefore, among these freshwater fish differences in TL content of the muscle may be due to differences in NL content. It has been shown in other fis that the lipid content of fis is influence by the content of NL [3].

Major fatty acid compositions in freshwater and marine fis are shown in Table 10.3. The predominant fatty acids of Chinese freshwater fis are 16:0, 16:1 n-7, 18:1 n-9, 18:2 n-6, and 22:6 n-3. Total monounsaturated fatty acids (MUFA) exceed 22% to the total fatty acids, and the major MUFA are 16:1 n-7, 18:1 n-9, and 18:1 n-7 [3]. The C20 and C22 MUFA are important in some marine fis such as sardine [5], herring [6], salmon [4,7], capelin [8], and sand lance [9]. They are also found at low levels in freshwater fish C20 and C22 MUFA are incorporated in lipids of marine fis that prey on Copepoda [6,9]. Ackman *et al*. [10] described that in the freshwater milieu there are no organisms comparable with the marine Copepoda and the major source of 22:1 acid in freshwater fis is the simple chain elongation from 18:1 n-9. The cultured freshwater fis in China have low levels of C20 and C22 MUFA.

Table 10.2 Popular Chinese freshwater fish sold in the Shanghai market

Family	Scientific name	English name
Cyprinidae	*Carassius auratus auratus*	Crucian carp
	Cyprinus carpio	Common carp
	Aristichthys nobilis	Big-head carp
	Hypophthalmichthys molitrix	Silver carp
	Ctenopharyngodon idellus	Glass carp
	Megalobrama amblvcephala	Blunt snout bream
Channidae	*Ophicephalus argus*	Snake-head fish
Percichthyidae	*Lateolabrax* sp.	Chinese sea bass
	Siniperca chuatsi	Chinese bass
Synbranchidae	*Monopterus albus*	Swamp eel

Table 10.3 Lipid content and major fatty acid composition (%) of total lipid in freshwater and marine fish

			Lipid (%)	20:1	22:1	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3	Reference
						% to total fatty acids					
Chinese freshwater fish	Crucian carp	Cultured	1.2	3.5	1.8	12.5	1.7	6.5	1.8	9.9	[3]
	Common carp	Cultured	1.9	1.2	0.1	9.0	2.3	3.8	5.2	5.3	[3]
	Big-head carp	Cultured	0.9	6.1	2.8	11.8	3.3	3.5	4.1	7.3	[3]
	Silver carp	Cultured	1.4	1.0	0.1	2.9	7.0	4.2	8.3	10.5	[3]
	Grass carp	Cultured	3.1	1.7	0.4	8.8	4.5	2.2	0.7	2.8	[3]
	Blunt snout bream	Cultured	4.7	3.1	1.4	5.1	0.7	1.4	0.2	1.4	[3]
	Snake-head fish	Cultured	1.1	1.4	0.2	3.9	3.4	4.9	2.1	14.8	[3]
	Chinese sea bass	Cultured	3.0	0.9	0.1	1.7	0.9	2.0	3.9	23.4	[3]
	Chinese bass	Cultured	3.8	1.1	0.1	21.1	2.7	2.0	0.7	2.9	[3]
	Swamp eel	Cultured	1.1	0.6	nd	5.0	2.6	6.3	1.0	4.7	[3]
	Common carp	Cultured	2.9	6.8	–	13.3	–	2.8	4.5	9.3	[13]
Japanese freshwater fish	Common carp	Wild	1.2	5.1	–	7.9	–	10.2	6.4	5.9	[13]
	Crucian carp		2.5	1.9	0.4	2.5	3.8	2.1	9.7	5.6	[17]
	Catfish		3.8	–	–	6.2	5.8	4.6	3.9	3.4	[13]
	Tilapia		5.3	2.3	0.5	11.9	0.8	1.1	0.7	6.2	[17]
	Ayu sweet fish	Cultured	7.9	3.4	1.6	8.4	0.9	0.4	2.8	6.9	[17]
	Ayu sweet fish	Wild	2.4	0.3	0.1	3.4	13.2	0.8	4.8	3.2	[17]
	Rainbow trout	Cultured in freshwater	4.6	5.8	3.0	10.0	1.1	0.7	3.9	15.5	[17]

(Continued)

Table 10.3 (Continued)

		Lipid (%)	20:1	22:1	18:2 n-6	18:3 n-3	20:4 n-6	20:5 n-3	22:6 n-3	Reference
					% to total fatty acids					
Marine fish	Rainbow trout	Cultured in sea	14.7	5.9	4.6	3.2	0.7	0.6	5.4	11.8	[17]
	Chum salmon		4.1	13.9	10.9	1.2	0.7	0.4	6.5	12.5	[17]
	Atlantic salmon	Cultured	16.1	8.0	8.3	3.1	1.1	0.6	6.9	11.2	[17]
	Yellowfin tuna		0.4	1.1	0.3	1.3	0.3	4.9	4.2	27.7	[17]
	Big-eye tuna		1.2	3.0	0.7	0.8	0.2	3.1	4.6	22.4	[17]
	Yellowtail	Young cultured	18.2	6.4	5.1	3.2	1.0	1.0	7.2	12.8	[17]
	Skipjack	Caught in autumn	6.2	2.9	2.6	1.8	0.9	1.8	8.5	20.7	[17]
	Red sea bream	Cultured	10.8	4.3	3.2	5.6	1.0	0.8	7.1	10.5	[17]
	Red sea bream	Wild	5.8	3.1	2.1	1.1	0.5	1.9	6.7	13.8	[17]
	Bastard halibut	Cultured	3.7	5.5	4.4	2.1	0.8	1.1	7.7	15.9	[17]
	Bastard halibut	Wild	2.0	4.8	3.6	1.0	0.5	2.9	8.2	19.0	[17]
	Walleye pollock		0.2	2.5	1.4	0.8	0.3	1.9	18.0	25.7	[17]
	Japanese sea bass		4.2	2.8	1.9	1.4	0.7	1.7	9.0	11.9	[17]
	Japanese pilchard		13.9	3.1	1.8	1.3	0.9	1.5	11.2	12.6	[17]
	Pacific saury		24.6	17.6	26.0	1.4	1.1	0.5	4.6	8.6	[17]

Abbreviation: nd, not detected.

This suggests that their diets do not contain materials such as marine fish meal from the clupeid family of fish which enhances accumulation of C20 and C22 MUFA [11,12].

The content of polyunsaturated fatty acids (PUFA) from Chinese freshwater fish range from 10 to 45% [3]. The n-3 PUFA, such as eicosapentaenoic acid (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3), have been considered as useful for human health. The content of 20:5 n-3 and 22:6 n-3 in the fatty acids of four species of Chinese freshwater fish (blunt snout bream, grass carp, Chinese bass, and swamp eel) was between 0.2 and 1.0% and between 1.4 and 4.7%, respectively. The level of 20:5 n-3 and 22:6 n-3 is comparatively lower than those of marine fish However, in another six species (crucian carp, common carp, big-head carp, silver carp, snake-head fish and Chinese sea bass), 20:5 n-3 and 22:6 n-3 contents were 1.8 to 8.3 and 5.3 to 23.4%, respectively. Chinese sea bass contained 23.4% of 22:6 n-3 in the total fatty acids. The content of long-chain PUFA such as 20:4 n-6, 20:5 n-3, and 22:6 n-3 were higher in PL, whereas 18:2 n-6 was higher in NL [3].

Kojima *et al*. [13,14] determined the fatty acid composition of some freshwater fish in Lake Biwa in Japan. In cited studies, the content of 18:2 n-6 of freshwater fish was higher than in marine fish The content of 18:2 n-6 in Chinese freshwater fish ranged from 1.7 to 21.1% of the total fatty acids. The 18:2 n-6 contents were higher than those of marine fish but similar to the data of Japanese [13,14] and Ethiopian freshwater fish [15]. Chinese bass contained 21.1% of 18:2 n-6, while the content of 20:5 n-3 (0.7%) and 22:6 n-3 (2.9%) was very low. Similarly, Mississippi farm-raised channel catfish contained a high level of 18:2 n-6 (12%) and a low level of 20:5 n-3 (0.4%) and 22:6 n-3 (1.2%) [16]. On the other hand, Chinese sea bass is originally a marine species, and thus contains high amounts of 20:5 n-3 and 22:6 n-3, and a low content of 18:2 n-6, similar to Japanese sea bass [17]. Cultured rainbow trout in fresh water contained a higher level of 18:2 n-6 than that of those cultured in the sea. Cultured marine fish also contained higher levels of 18:2 n-6 than wild marine fish The 18:2 n-6 is a typical fatty acid in freshwater and cultured marine fish

The contents of 18:2 n-6, 20:5 n-3, and 22:6 n-3 of Indian freshwater fish (*Callichrous pabda*) [18] are similar to Chinese sea bass, but the content of 20:4 n-6 was higher than all Chinese freshwater fish reported by our group [18].

Liu [19] examined the fatty acid composition of five species of Chinese freshwater fish belonging to the Cyprinidae and had high contents of 18:3 n-3 in grass carp (32–35%). However, the content of 18:3 n-3 was modest (0.7–7.0%) in 20 species of Chinese freshwater fish [3], with the highest content of 18:3 n-3 being in silver carp (7.0%) (Table 10.3).

Diet has a major effect on the fatty acid composition of fish lipids [20]. Most Chinese freshwater fish are cultured fish therefore it would be possible to control fatty acid profile of Chinese freshwater fisherie resources by their diet.

10.3   The effect of dietary fatty acid composition in cultured freshwater fish

The importance of dietary intake of long-chain highly unsaturated n-3 fatty acids, such as EPA and DHA, for human health is well known. Marine fish are good sources of these n-3 fatty acids. Some freshwater fish species contain a relatively high amount of n-3 PUFA, similar to marine fish Chinese carps (without grass carp) are rich in n-3 PUFA (Table 10.3), whereas n-3 PUFA in blunt snout bream is only in trace amounts. Fatty acid composition of the feed for cultured blunt snout bream is shown in Table 10.4, suggesting that lower levels of n-3 PUFA in this fish would be due to a lesser amount of n-3 PUFA in the diet.

Table 10.4   Fatty acid composition (%) of diet for cultured blunt snout bream.
Adapted with permission from Kaneniwa [21]

Fatty acid	Rapeseed meal	Barley	Commercial diet
16:0	8.6	24.8	16.2
18:0	1.6	1.5	1.8
16:1 n-7	0.9	0.3	0.6
18:1 n-9	42.4	10.9	34.4
18:1 n-7	5.5	0.7	2.3
20:1 n-9	0.3	0.6	1.2
22:1 n-11	0.1	1.6	3.5
18:2 n-6	30.9	50.3	30.8
18:3 n-3	5.9	3.2	4.4
20:4 n-6	nd	nd	nd
20:5 n-3	nd	0.6	nd
22:6 n-3	0.1	nd	nd

Abbreviation: nd, not detected.

A feeding experiment was conducted using juvenile blunt snout bream [21], because this fis is easily affected by the dietary lipids. Fish oil extracted from whole fis body of silver carp was supplemented to the commercial diet. Dietary oil supplementation improved the growth of the fis (Fig. 10.1). Fatty acid compositions of juvenile blunt snout bream were influence by the different oil sources (Table 10.5). The supplementation of silver carp oil increases the PUFA content of the fis [21].
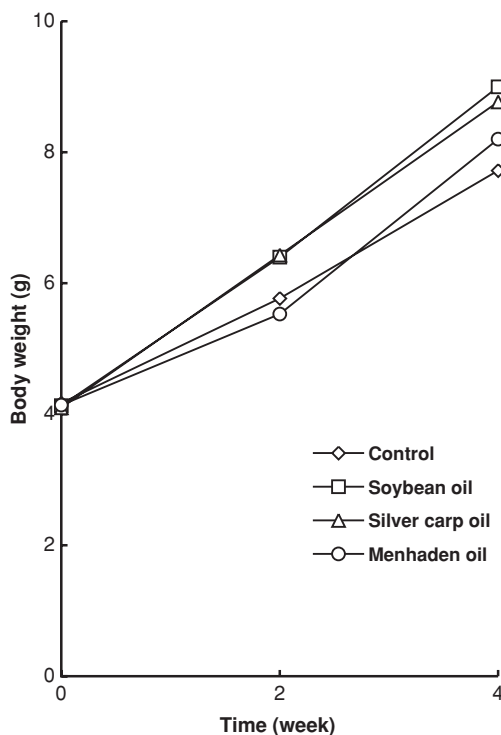
Fig. 10.1   Growth of juvenile blunt snout bream. Adapted with permission from Kaneniwa [21].

Table 10.5 Fatty acid composition (%) of total lipid from blunt snout bream fed with different oil sauces. Adapted with permission from Kaneniwa [21]

Fatty acid	Control	Soybean oil	Silver carp oil	Menhaden oil
Saturated	28.6	19.9	26.4	29.8
18:1 n-9	24.6	22.4	19.8	17.7
18:2 n-6	12.9	29.9	11.5	10.5
20:5 n-3	1.1	1.0	3.2	3.5
22:6 n-3	4.9	4.2	7.9	9.7

10.4 Enzymatic hydrolysis of lipid in the muscle of freshwater fish

Enzymatic hydrolysis of lipid in fis muscle has been reported in some of the both lean and fatty fis that include cod, skipjack, carp, sardine, and rainbow trout [22–27]. Free fatty acids (FFA) accumulate in muscle lipids from the enzymatic hydrolysis. FFA degrade the quality of fis muscle [28–30]. Enzymatic hydrolysis that occurs in the muscle of Chinese silver carp has been reported [3].

Changes in the lipid classes of silver carp muscle during storage at $20°C$ for 8 days are shown in Fig. 10.2 [3]. During storage for 8 days, FFA increased from 0 to 28%, PL decreased from 89 to 60%, but TAG contents did not change. These phenomena were inhibited by heating the muscle. Thus, it is suggested that hydrolysis of lipids by phospholipase had
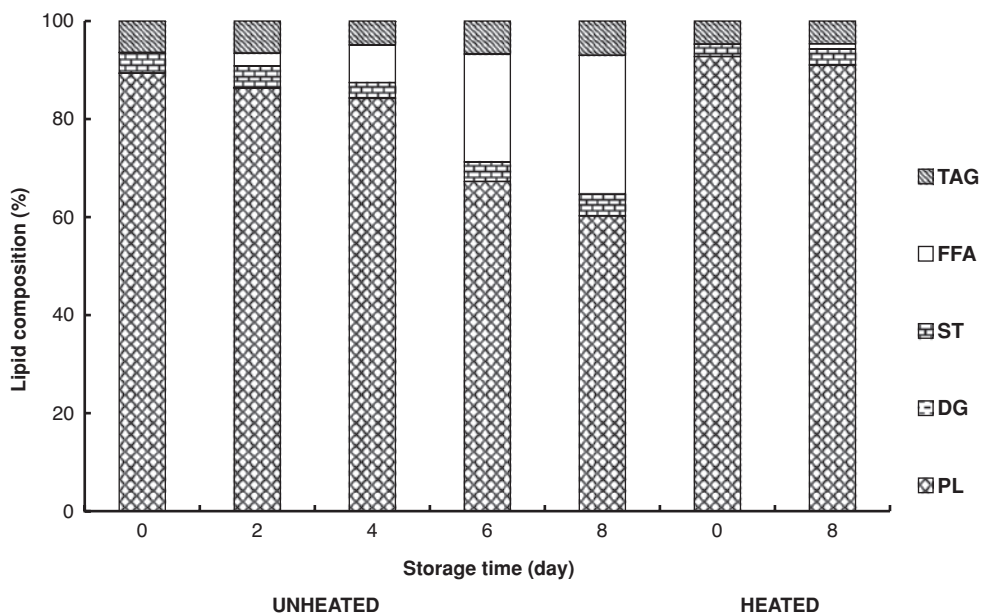
Fig. 10.2 Changes in the lipid class of silver carp muscle during storage at $20°C$. Adapted from Kaneniwa *et al.* [3], with kind permission from Springer Science+Business Media. Heated: Heated in boiling water for 10 minutes. TAG, Triacylglycerols; FFA, Free fatty acids; ST, Sterols; DAG, Diacylglycerols; PL, Polar lipids.

taken place in silver carp muscle and phospholipase in heated muscle were deactivated by denaturation of the enzyme. The fatty acid composition of TL and TAG did not change throughout the storage. However, some changes of PL fatty acid composition were observed. Levels of 20:5 n-3 decreased, while that of 22:6 n-3 increased during the storage. It is suggested that the 20:5 n-3 was removed from PL by hydrolysis, but the 22:6 n-3 remained in PL, then levels of the 20:5 n-3 in PL decreased and that of 22:6 n-3 increased. The 20:5 n-3 was evidently more prone to hydrolysis than the 22:6 n-3.

In most previous studies, enzymatic lipid hydrolysis was carried out at a low temperature (<5°C) and long-term storage (>1 week). However, these conditions are not suitable for purificatio of the enzymes. Thus, we examined FFA formation during enzymatic hydrolysis in the muscle of some Chinese freshwater fis at the high temperature of 37°C and during a short-term incubation of less than 6 hours [31]. The muscles from three species of Chinese freshwater fis (grass carp, big-head carp, and silver carp), including white and dark tissue, were homogenised with four volumes of distilled water and incubated at 37°C for 2, 4, and 6 hours. During incubation, the content of FFA increased, while that of PL decreased in all samples; however, the TAG contents did not change (Fig. 10.3). These results indicate that the muscle PL of these Chinese freshwater fis was hydrolyzed during incubation, and hydrolysis was mainly caused by phospholipase in the muscle. Increments of FFA per 100 g of muscle were 4.4 mg (grass carp), 41.7 mg (big-head carp), 11.7 mg (silver carp), and 114 mg (skipjack). In Chinese freshwater fish lipid hydrolysis was observed in their muscle, but increments of FFA were lower than those of skipjack [31].
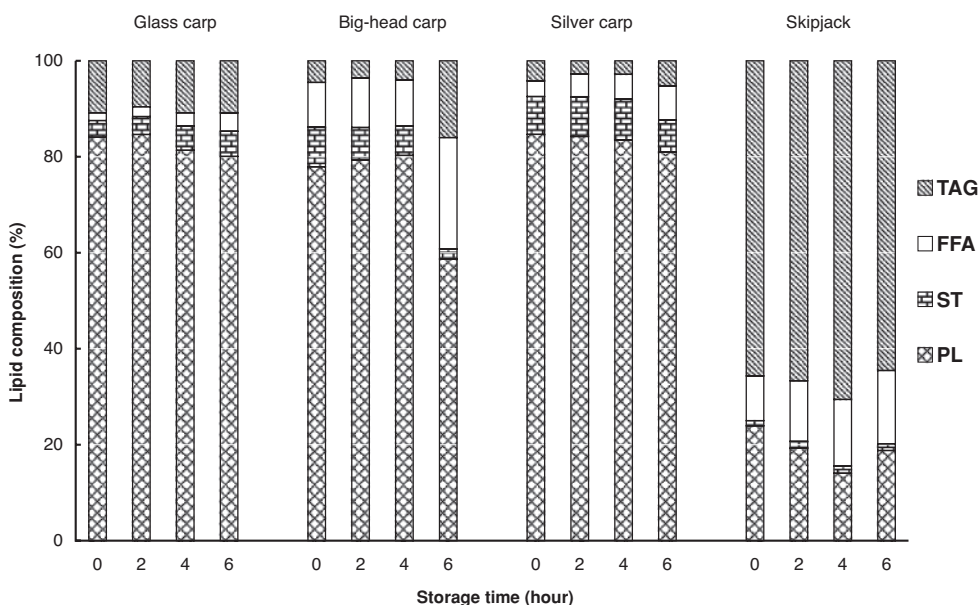
Fig. 10.3 Changes in the lipid class of homogenates prepared from muscle of Chinese freshwater fish and skipjack during incubation at 37°C. Adapted from Kaneniwa *et al*. [31], with permission of the American Fisheries Society. TAG, Triacylglycerols; FFA, Free fatty acids; ST, Sterols; PL, Polar lipids.

10.5   Quality of frozen surimi from freshwater fish meat

Frozen surimi is an intermediate foodstuff with potential for long shelf-life, for distribution over a wide area, and for the production of various processed foods. Hence, a vast quantity of cultured freshwater fis may be produced and utilised as new materials in surimi and other processed forms in the near future.

Luo *et al*. [32] compared the gel properties of surimi from Alaska pollock and three freshwater fis species. The gel forming abilities of freshwater fis surimi were inferior to that of Alaska pollock surimi, but freshwater fishe can be utilised as materials for surimi and processed foods by processing at appropriate gel forming conditions. The protein concentration affected the gel strength most significantl , but heating temperature and period were found not to be the main factors influencin the gel strength [32].

Wang *et al*. [33] classifie eight species of freshwater fis surimi into two types according to their gel-forming properties. One type of surimi, including silver carp, big-head carp, Chinese snake-head, and blunt snout bream, exhibited easy setting, low resistance to gel collapse, high enhancement ability with a two-step heating, and narrow optimum heating temperature and time span. These characteristics are similar to those of Alaska pollock surimi. In contrast, other types of surimi, including those from tilapia, grass carp, mud carp, and common carp, are difficul to set, highly resistant to gel collapse, have no enhancement ability with two-step heating, and have wide optimum heating temperature and time span.

The gelling properties of silver carp surimi are also affected by seasonal changes, with the setting ability of surimi gel being higher in winter and lower in summer. In addition, thermal stability of silver carp myofibrilla protein is higher in summer and lower in winter. Therefore, the setting ability of surimi gel is evidently affected by the thermal stability of myofibrilla protein, thus a higher inactivation rate of myofibrilla protein leads to a higher rate of setting [33]. In recent studies, it was elucidated that seasonal differences in the gel forming ability of silver carp was derived from seasonal differences in thermal stability of myosin in surimi [34], and seasonal expression of two types of myosin with different thermostability in silver carp muscle are demonstrated [35].

10.6   Conclusions

Some of the Chinese freshwater fis are regarded as good resources of PUFA. On the other hand, a high activity of lipid hydrolysis in muscle of Chinese freshwater fis suggests that prevention of lipid oxidation and hydrolysis is necessary for preservation and processing of these freshwater fish Although the gel forming abilities of Chinese freshwater fis are inferior to that of Alaska Pollock, these freshwater fis can be utilised as raw material for surimi production with consideration of appropriate gel forming conditions or seasonal varieties.

10.7   Acknowledgements

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11 Texture measurements in fish and fish products

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11.1 Introduction

Texture is one of the most important parameters that determines the overall quality perception of fish Seafood is normally more perishable than other muscle foods. Soft fles often leads to reduced acceptability by the consumer [1], and to quality downgrading in the fis processing industry [2]. Also, unlike beef, post-mortem tenderization or autolysis of fis muscle is highly undesirable to processors and consumers. In most terrestrial myosystems, it is advantageous to increase meat softening. The situation is opposite for most seafoods, since soft muscle texture lowers fille yield and appearance, resulting in problems for the fis processing industry [3–8]. Fish muscles have softer texture compared to land animals, because they have one-tenth collagen and less cross-links than muscle from terrestrials [3]. The collagen content and its properties contribute to the texture of raw fish but it is far less relevant for textural properties of cooked fis [9].

Today, increasing importance is giving to animal handling stress in the late ante-mortem period, because it affects the rate and the extent of muscle metabolism during the early post-mortem period and consequently affects muscle quality. Choosing the most suitable method for ante-mortem handling is an important step to ensure fis fles of good quality [10,11]. Exercise, activity, or stress prior to slaughter also contribute to fille softening and gaping [12,13]. Thus, there has been increasing interest in regulating the methods of killing fis [14].

Texture is a difficul term to defin and many different definition of the word exist for the consumer as well as food technologists, since it is an important attribute for mechanical processing of fillet by the food industry [3]. It is a complex sensory phenomenon, consisting of a group of properties derived from the structure of the food, and can be described by physical properties (mechanical or rheological) [15] and instrumental analysis cannot fully simulate the overall experience of texture [7].

Texture of fis meat is influence by several factors, such as fis species, age, and size of the fis within the species, fat content and localization in muscle, amount and properties of the proteins and connective tissue, and handling stress before slaughter. From the moment the fis is killed, post-mortem factors become important; these include rate and extent of pH decline, rigor mortis, rate and extent of proteolysis causing breakdown of myofibril and

connective tissue, degradation of nucleotides, and temperature during and length of storage period [13,16,17]. The post rigor fis muscle becomes softer with increasing storage on ice [18–20]. Besides, the acidity of the muscle is probably the most important single factor affecting the textural properties of fis flesh which will depend on the pre-slaughter stress or activity of the fis [13,21]. The extent of the action of pH on texture of fis fles depends on the rate of post-mortem pH decline in the muscle; thus a rapid pH decline may cause soft texture and poor water-holding capacity of the meat, even when the ultimate pH is low [13,22,23]. Besides, fis meat quality is influence by the amount of muscle glycogen, as it determines the ultimate pH of the meat [3]. The muscle glycogen content may be lowered by ante-mortem stress (during capture of wild fis and netting/transport of farmed fish or starvation [3,5,9]. According to Haard [3] and Hallier *et al*. [8], farmed fis tend to have a softer, less preferable texture than free-living fish This chapter reviews texture measurements in fis and fis products.

11.2   Measurement of fish texture

Quantifying textural properties such as hardness, fracturability, cohesiveness, springiness, and resilience has always been difficul by either instrumental or sensory means. A wide variety of knives, spheres, and cylinders have been used to cut or press into fis fillets cutlets, or other pieces of given geometry, both parallel to and perpendicular to the muscle fibre [17,19,24–26]. Some methods that focus on less destructive action, such as compression, are recommended [6]. On the contrary, some destructive methods are recommended to determine the fis texture because they better reflec the destructive action of the mouth during mastication [7]. There is no ideal texture measurement equipment or system that can be universally recommended. Considering the fisher industry, the method mostly used is the finge method, whereby a person evaluates fis firmnes and elasticity by pressing a finge on the fis or fillet Thus, development of instrumental methods has made it possible to get more objective measurements of textural properties, other than the finge method, as well as avoiding the high costs of highly trained personnel for sensory evaluations.

11.2.1   Instrumental versus sensory methods

The usefulness of a method for objective texture measurement of foods depends largely on the correlation with sensory properties, which could be useful for quality control of the fisher industry and to predict consumer responses [13]. During the last decade, several articles have been published on instrumental texture analyses [7,25,27–29] and texture analyses using both sensory and mechanical measurements [15,17,30]. Many attempts, with varying results, have been made to establish a relationship between instrumental instruments and sensory evaluation, but unfortunately they do not consistently show a correlation. Only a few studies have correlated a variety of instrumental methods with sensory evaluated texture attributes. Examples are those of Bordeiras *et al*. [31] with rainbow trout, Schubring [6] with unfrozen Atlantic cod, and Morkore and Einen [7] with smoked salmon.

11.2.2   Raw and cooked fish products

The texture of raw and cooked fis products is important and attempts have been made to measure both. Although the history of fisherie began thousand of years ago, determining

fis quality by instrumental means is recent. Several attempts, until 1990, have been made to improve different mechanical methods for measuring texture of raw fillets however, they involved high cost, destructive sampling, low speed, and difficult in obtaining results. Botta [24] developed a patented method, a portable tester, for the rapid non-destructive measurement of raw Atlantic cod fillets measuring firmnes and resilience, and determining surface and thickness of the fillet with increasing force. A fille with a high texture index, hence a high fresh quality, has low deformation distance and a relatively large rebound distance.

The major method for measuring cooked seafood is shearing force, which although being effective to measure texture, has disadvantages such as destructive sampling, high costs, and slow speed [24]. However, other methods have been used such as double compression and punching. An extensive review on these fis products, either raw or cooked, has been published [32].

11.2.3 Sensory evaluation

Sensory methods of fis texture measurement are frequently used for experimental analyses [8,15,17]. The common tests are the Texture Profil Analysis, Quantitative Descriptive Analyses, and Anchored Descriptive Analyses. These methods, although time consuming, are expensive and complex and offer the opportunity to obtain a complete analysis as perceived by human senses. Sensory analysis of fis products has always been an important part of the production process, and although instrumental and chemical testing of fis texture have high scientifi value, both have limited value in assessing the fina quality of the product. Thus, complimentary sensory analysis is often necessary to determine quality parameters and shelf-life of seafood products [8,33]

11.2.4 Texture measurement of fish flesh

Classifying fis texture is difficul because of the non-uniformity of fis and shellfis structure, which is reflecte on both a small scale (e.g. flakiness and large scale (e.g. variation along the fis body). Furthermore, even after filletin a fish it maintains unique shape characteristics, making it difficul to prepare standard specimens for mechanical testing. In such cases, the possibility of accurate mapping of textural differences becomes questionable and, consequently, other accurate comparisons between species or individual fis are difficul [34–36].

11.3 Relevance of measuring texture in fish products

The totality of fis products entail aspects related to gastronomic delights, purity, nutrition, safety, consistency, fairness (in labelling, weigh or species), product value, and excellence. In the international fis trade, two of the more prominent aspects are safety and sensory quality [37], and texture is one of the most important parameters to measure. To be evaluated adequately, texture is a sensory property that requires highly trained panellists. When trained panellists are not available, instrumental texture measurements can serve as an alternative [38].

11.3.1 Firmness: a quality for good fish texture

The firmnes of raw muscle, a central aspect of meat quality, is a critical parameter that determines the acceptability of seafood products [25]. Instruments to measure texture do not have the human ability to evaluate and to interpret multivariate input [39]. If texture can be specifie in an early processing step, inferior quality in the fina product can be reduced [36,38,40,41].

11.3.2 Muscle structure of fish flesh

Muscle fibr is a very important determinant of the textural characteristic of fis flesh The analysis of muscle fibr and its components, namely myofibrilla proteins and fat, is outside the scope of this chapter. Besides, excellent research has been done to study fis muscle [42,43]. These studies provide an important basis for applied seafood research on fis flesh It has been observed that the increase of muscle fibr size leads to a decrease in firmnes and to an increase in the coarseness of the fles [34,44–46], and in turn such fir fibr size depends on where the fis grows, if in the wild or in a farm [34]. Firmness also depends on whether the fis is caught in the winter or in the summer [38]. In addition, many other variables that must be taken into account to produce fis fles of uniform quality are:

- variations of the muscle at different fishin locations, which according to Love [47] may be due to geographical, seasonal, and feeding factors, the orientation of the fla es within the specimen, post-mortem biochemical factors, and the filletin process itself;
- intrinsic factors (species, compositions, and size) and post-mortem factors (glycolysis, rigor mortis, gaping of fis muscles, changes of toughness, skeletal attachment, and effect of temperature) [48];
- frozen storage affects the ultra structure and texture of fis muscle [16].

11.3.3 Muscle cell biology

In fish there are uncertainties regarding the underlying mechanisms and factors that contribute to the post-mortem softening of the fles [11]. A large number of studies have investigated the effects of muscle cellularity on ante-mortem and post-mortem factors, which impact the fles quality. In a study with fi e different species, Hatae *et al*. [44] demonstrated that fis fillet with fir texture after cooking had thin muscle fibre containing a considerable amount of heat coagulating material, whereas species having soft texture had thick muscle fibre with little heat coagulating material. Hurling *et al*. [49] analyzed seven fis species confirmin a significan relationship between fibr diameter and sensory firmnes of cooked fish Johnston *et al*. [45] determined firme texture and better coloration in a slow-growing salmon strain compared with a fast-growing strain. Texture and colour differences were attributed to variation in muscle cell size. Moreover, the relationship among fille texture, fibr arrangement, and density is not consistent [17,34,40].

   There is a gap in the knowledge about why pre-mortem factors such as the energy level through stress should have such an impact on the post-mortem softening of the fles [11]. Some attempts have been made to suggest that some mechanisms other than energy metabolism and rigor mortis are the source for accelerating the post-mortem tenderization. Nordgreen *et al*. [14] and Roth *et al*. [21] used electrical stimulation as a stunning method, on Atlantic salmon fillets to reverse the stress response.

11.3.4   Physical exercise

Contradictory results were published concerning the action of swimming exercise on fis texture. Swimming exercise increased the fles firmnes in both Atlantic salmon and sea bream [50,51]. Johnston *et al*. [45] found that firmnes (taste panel) increased the fibr density in Atlantic salmon. On the contrary, neither Sigurgisladottir *et al*. [28] nor Bjornevik *et al*. [46] found a relationship between muscle fibr diameter and texture as measured by shear force. Now, there is increasing evidence that exercise prior to slaughter contributes to softening and gaping of fis fles [12]. Comparing the results from different studies on texture quality is complicated because of the use of different instrumental analysis on raw fis or sensory analysis on cooked fish due to the inconsistent correlation between these two methods [4,46].

11.4   Textural measurements of fish products

Various instrumental methods are used to determine textural properties of fis flesh The type of instrument used depends on whether the product is raw or cooked. However, some methods are used in both cooked and raw products. The mechanical methods, and the importance of texture as a quality attribute of various fis products such as raw fillets cooked fillet and patties, and sausages were reviewed [15,32,52]. Thus, for raw fis fillet and cooked seafood other than hand-held probes, the most common instrumental methods used are the Automatic Penetrometer, Instron Universal Testing Machine (with Ottawa texture measuring wire shear cell or Kramer shear compression cell), Stevens Compression Response Analyzer, Botta's Method, Cylindrical Plunger (0.8 cm dia), Rheological Gel, TA.XT2 Texture Analyzer, and Differential Scanning Calorimetry. Various types of shear cells are attached to the equipment, such as a four-blade Kramer shear cell or a ten-blade Kramer shear cell.

A considerable amount of research has been carried out to measure the texture of fis flesh using the methods mentioned above. Some new methods are being researched (cheapest, easiest, and quickest) for the fis industry to measure texture and to compare the results with sensory evaluation in order to fulfi the consumer's taste. In recent years, research has been conducted to measure how fis fles varies, depending on being stored in ice or fresh, on starvation and re-feeding of fish or on being farmed or wild, etc. Thus, there are a lot of variables to study, including sex, weight, and age of fish season of the year, catching of wild fish types of instrument and method used, and variation in sensory evaluation. On the other hand, texture results depend on the region of fis fles measured; hardness as well as shear force increase from head to tail [6,25,53]. Thus, measuring fis texture is not an easy task, when comparison is made with fis fles from the same species from different fis processing plants.

Table 11.1 summarizes some relevant topics about texture measurements that are being studied, as a way of improving the control of fis product quality. Most research was carried out using a TA.XT2 texture analyzer [32].

11.5   Conclusions

Texture is a complex characteristic of aquatic foods and can be evaluated by using sensory and instrumental methods. Texture measurement should include both methods, and be

Table 11.1 Some examples of instrumental analyses used for determining texture at different treatment conditions of fish flesh

Species	Conditions	Instrument used	Result	Reference
Gadus morhua	Ice storage	Zwick hardness tester	Good correlation between firmness and sensory assessment.	[6]
Sparus aurata	–	Texture analyzer (TA.XT2)	Sensory, freshness, and texture relationships.	[53]
Labeo rohita	–	Texture analyzer (Ta-Hdi)	Decreasing skin hardness.	[23]
Salmo trutta	Starvation and re-feeding	Universal testing machine (Instron 5444)	No differences between starved and refed.	[9]
Salmo salar	Aquaculture	Texture Analyzer (TA.XT2)	Measurement must be taken at the same fish location.	[41]
Atlantic salmon	–	Texture Analyzer (TA.XT2)	No differences in hardness and fracturabilty.	[35]
Silurus glanis	–	Universal testing machine (Instron 5444)	Softer raw and cooked fillets with farming time and water temperature.	[8]
Atlantic salmon	Raw and smoked fillets	Texture Analyzer (TA.XT2)	Potential of predicting texture.	[7]
Farmed cod fillets	Sustained swimming exercise	Texture Analyzer (TA.XT2)	No relationship between fibre dia and shear force.	[46]
Salmon fillets	Stereoscopy: A new method to detect different treatments	Brookfield texture analyzer (LTRA 1500)	Fillet treated with pepsin and papain showed low firmness values.	[36]
Smoked salmon	Increasing brine concentration	Texture Analyzer (TA.XT2)	Increased hardness and decreased elasticity.	[54]
Salted cod	Freezing/thawing prior to salting	Texture analyzer (KGS)	Increased firmness of salt cured product than chilled salted.	[55]
Tuna fillets	Pressure treatment	Universal texture machine	Increased firmness and springiness.	[56]

reproducible to better understand changes that occur in fish fles and processed products. Extensive studies on the mechanical properties of fish fles and products have led to the development of instruments that accurately record the various movements of mastication as well as provide an objective instrumental analysis. This has allowed researchers a variety of instrumental tests so that there is not a complete reliance on sensory tests for routine evaluation. Instrumental and sensory tests can be complimentary and give a better overall understanding of texture characteristics. Unfortunately, studies on comparison between instrumental and sensory measurements are scarce, since they require different kinds of expertise. However, excellent research is being done, taking into account many factors that influenc fis texture, such as farming conditions, exercise, starving and re-feeding of fish brine, salting of fish ice storage, muscle structure, and muscle proteins, etc. The goal is to improve fis texture in order to satisfy the consumer's preference. Although several physical methods have been

described with different types of instrumentation, it is essential to maximize the benefit and minimize the limitations of those methods and decide which is best, depending on type of species, product, and intended results.

11.6 Acknowledgements

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12 Quality and safety of packaging materials for aquatic products

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12.1 Introduction

Food packaging like any other packaging is an external means of preserving food during storage, transportation, and distribution and has to be provided at the production centre. Unlike many other manufactured consumer products, the packaging needs of food and food products, particularly fish are very complex because of the intrinsic characteristics and the need to preserve them while in the package. This chapter describes quality requirements and appropriate packaging materials for aquatic products and also on the safety of packaging materials for food contact application.

12.2 Packaging materials

Figure 12.1 shows a range of packaging materials used for aquatic products. Their advantages and disadvantages are detailed in a subsequent section of this chapter.

12.2.1 Glass containers

Glass containers such as bottles, jars, tumblers, and jugs have been used for many centuries and still are important in food packaging. It is strong, rigid, and chemically inert and does not appreciably deteriorate with age. It is an excellent barrier to solids, liquids, and gases and gives excellent protection against odour and fl vour contamination. Transparency of glass provides product visibility and can also be moulded to a variety of shapes and sizes. However, it has disadvantages such as fragility, photo-oxidation, and weight [1].

12.2.2 Metal cans

Cans are traditionally used for heat sterilized products and common types are made from standard tin plate, light weight tin plate, double reduced tin plate, tin free steel, and vacuum deposited aluminium on steel and aluminium. For packing food products, they are coated inside to give the desirable properties, such as acid resistance and sulphur resistance. Metal
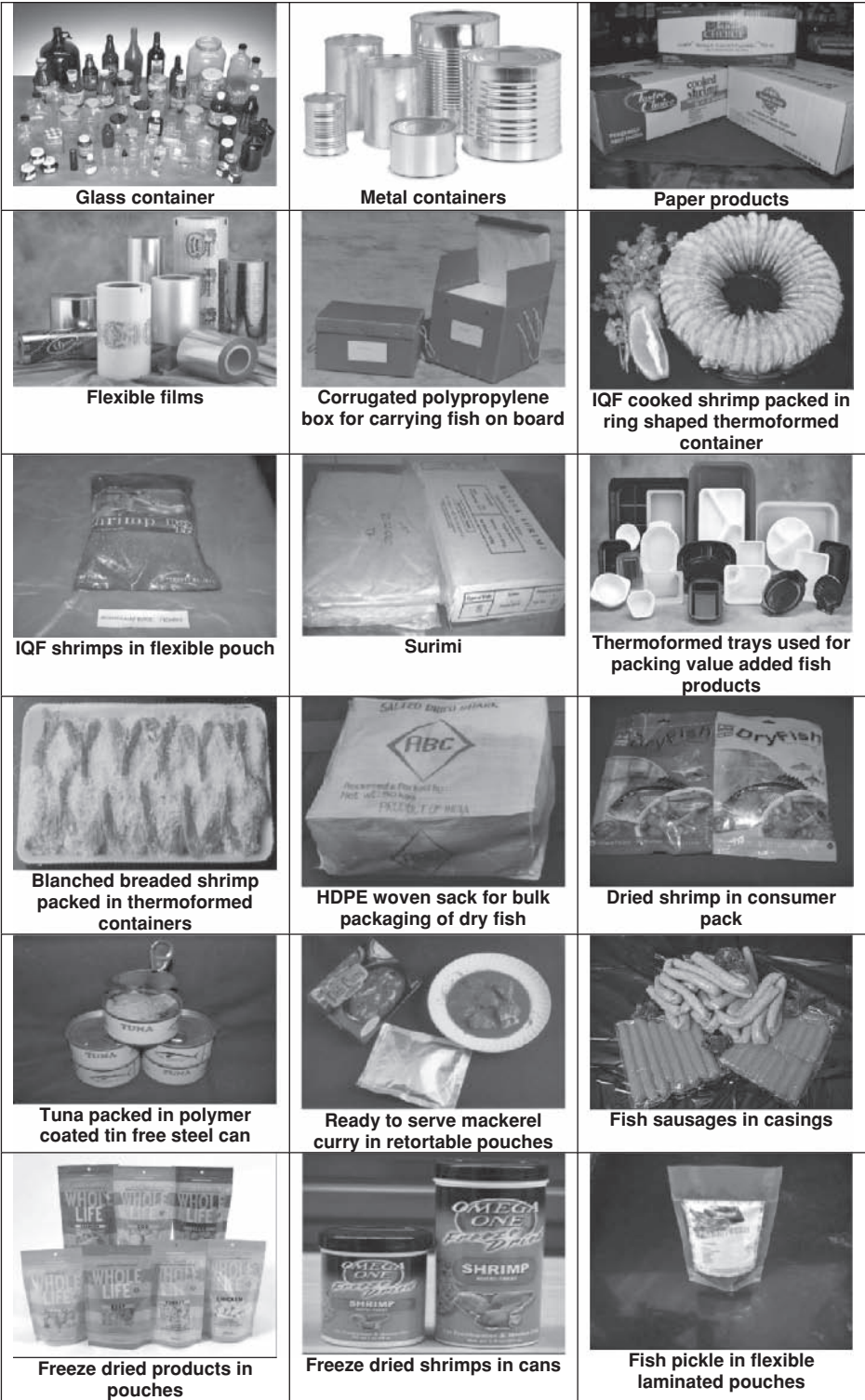
Fig. 12.1   A range of packaging materials used for aquatic products. For a colour version of this figure, please see the colour plate section.

cans are advantageous because of superior strength, high speed of manufacture, and easy fillin and closing. Disadvantages of metal cans are weight, difficult in reclosing, and disposal. Open top sanitary tin cans are used for the manufacture of fis cans. Generally, a low metalloid content steel with a phosphorous content of 0.02% (called Type MR Quality steel) is used in the manufacture of tin cans [1].

12.2.3 Paper

A significan portion of packaged foods is stored and distributed in packages made out of paper or paper based materials. Because of its low cost, easy availability, and versatility, paper is likely to retain its predominant position in the packaging industries. However, paper is highly permeable to gases, vapours, and moisture and loses its strength when wet. Ordinary paper is not grease and oil resistant, but can be made resistant by mechanical processes during manufacturing. White board prepared from sulphite pulp is used for making duplex cartons, which are used as inner cartons for the export of frozen shrimp. The main characteristics of the board are thickness, stiffness, ability to crease without cracking, whiteness, and suitability for printing. The inner carton should hold and freeze the products without distortion, close properly, and fi the corrugated box in the proper manner. The ink used for printing the carton should be free from toxic metals such as lead and chromium. The carton should have details printed on the exterior such as name and address of the exporter, brand name, type of product, net content, and size grade. The shipping container consists of corrugated fibr board made up of kraft paper. The quality of kraft paper used in the manufacture of corrugated fibr board plays a very important role in packaging functions. It should have good mechanical strength and should not weaken due to deposit of moisture caused by temperature fluctuatio during loading, unloading, and other handling stages [2].

12.2.4 Cellophanes

Cellophane was the firs commercial film Cellophane is manufactured from highly purifie cellulose derived from bleached sulphite pulp. By incorporating various coatings and modifications over 100 different grades of cellophane are now available [3].

12.2.5 Polyethylene

Low-density polyethylene (LDPE) is widely used by the packaging industry as it possesses qualities such as transparency, water vapour impermeability, heat sealability, chemical inertness, cheapness, and resists temperatures between -40 to $+85°C$. Permeability to organic vapours, oxygen, and carbon dioxide is high and it has poor grease barrier properties. It is commonly used as an inner wrap in the export of frozen shrimp/fish The inner wrap should be of food grade and fl xible at low temperatures.

High-density polyethylene (HDPE) resins are produced by a low-pressure process and the density is around 0.95 g/cm^3. HDPE possesses a much more linear structure than LDPE and has up to 90% crystallinity, compared with LDPE, which has only 50%. HDPE is stronger, thicker, less fl xible, and more brittle than LDPE and has lower permeability to gases and moisture. It has a higher softening temperature ($121°C$) and can, therefore, be heat sterilized. High molecular weight HDPE (HM-HDPE) has very good mechanical strength, less creep, and better environmental stress crack resistance properties [4].

Linear LDPE (LLDPE) is produced by a low pressure process. Normal LDPE has many − C_5H_{11} side chains. These are absent in LLDPE, allowing the molecules to pack closer together to give a very tough resin. It is virtually free of long chain branches but does contain numerous short side chains. Generally the advantages of LLDPE over LDPE are improved chemical resistance, improved performance at low and high temperatures, higher surface gloss, higher strength at a given density, and a greater resistance to environmental stress cracking. LLDPE shows improved puncture resistance and tear strength. The superior properties of LLDPE have led to its use in new applications for polyethylene, as well as the replacement of LDPE and HDPE [5].

12.2.6   Polypropylene (PP)

Polypropylene is produced by the polymerization of propylene. These film are stronger, rigid, and lighter than polyethylene and have permeability of about a quarter to half of polyethylene. Four types of PP film are used in the food industry, as follows.

- Cast PP: this is extruded, non-oriented film characterized by good stiffness, grease and heat resistance, and also has a good moisture barrier. However, it is not a good gas barrier.
- Oriented, Heat set PP (OPP): orientation can be in one direction (unbalanced) or in two directions (balanced). The resulting fil   has high stiffness, good moisture vapour transmission, and can withstand low temperatures. One drawback is its low tensile strength.
- Coated PP: these newer types of PP are available as heat seal coated or saran (PVDC) coated. They are used when moisture and gas protection is necessary.
- Composite PP: this has outer polyethylene plies around a PP core. This material can be readily heat-sealed.

Practically all these materials have been in use for packaging different products due to their advantages. However, plastics used in recent years have many disadvantages. Some of the chemical adjuvants used in the manufacture of plastic materials may be toxic in nature and can be transferred to the food when the package is in contact with the food material. However, plastics have the advantage that most of them possess excellent physical properties such as strength and toughness. They are light in weight and fl xible, and also resistant to cracking. A wide range of polymers are now available for conversion into diverse types of plastic packaging materials. However, the requirements with a particular food may not be met with a simple material, as it may not possess all the desired properties. In such cases, copolymers or laminates consisting of two or more layers of different polymers having different properties can be used [6].

12.2.7   Polystyrene (PS)

Manufactured from ethylene and benzene, the PS is a good barrier to gases and a poor barrier to water vapour. New applications of PS involve co-extrusion with barrier resins such as ethylene vinyl alcohol (EVOH) and poly vinylidene chloride copolymer to produce thermoformed, wide mouthed containers, and multi-layered blow moulded bottles for shelf stable food products. To overcome the brittleness of PS, synthetic rubbers can be incorporated at levels generally not exceeding 14% by weight. High impact PS is an excellent material for thermoforming. Co-polymerization with other polymers such as acrylonitrile butadiene improves the fl xibility. It is also used as a breathing fil   for packaging fresh produce. Since

it is crystal clear and sparkling, PS is used in blister packs and display covers. These materials have low heat sealability and often tend to stick to the jaws of heat sealers [7].

12.2.8   Polyester

Polyester can be produced by reacting ethylene glycol with terephthalic acid. Polyester film s outstanding properties as a food packaging material are its great tensile strength, low gas permeability, excellent chemical resistance, light weight, elasticity, and stability over a wide range of temperatures (–60 to 220°C). The latter property has led to the use of polyethylene terephthalate (PET) for boil-in-the-bag products, which are frozen before use and as oven bags where they are able to withstand cooking temperatures without decomposing [7].

Although many film can be metallized, polyester is the most commonly used. Metallization results in a considerable improvement in barrier properties. Reduction in water vapour transmission rates by a factor of 40 and oxygen permeabilities by over 300 is normally obtained. A fast growing application for polyester is oven-proof trays for frozen food and prepared meals. They are preferable to foil trays for these applications because of their ability to be microwaved without the necessity for an outer board carton.

12.2.9   Polyamides (nylon)

Polyamides are condensation products of diacids and diamines. The firs polyamide produced was nylon-6,6 made from adipic acid and hexamethylene diamine. Various grades of nylons are available. Nylon-6 is easy to handle and is abrasion-resistant. Nylon-11 and nylon-12 have superior barrier properties against oxygen and water and have lower heat seal temperatures. However, nylon-6,6 has a high melting point and so it is difficul to heat seal. Nylons are strong, highly crystalline materials with high melting and softening points. High abrasion resistance and low gas permeability are other characteristics [8].

12.2.10   Polyvinyl chloride (PVC)

This monomer is made by the reaction between acetylene and hydrochloric acid. It must be plasticized to obtain the required fl xibility and durability. Films with excellent gloss and transparency can be obtained by using the correct stabilizer and plasticizer. Thin plasticized PVC fil   is widely used in supermarkets for the stretch wrapping of trays containing fresh red meat and produce. The relatively high water vapour transmission rate of PVC prevents condensation. Oriented film are used for shrink-wrapping of fresh products. Unplasticized PVC as a rigid sheet material is thermoformed to produce a wide range of inserts from chocolate boxes to biscuit trays. Unplasticized PVC bottles have better clarity, oil resistance, and barrier properties than those made from polyethylene. They are useful for packing a wide range of foods, including fruit juices and edible oils [8].

12.2.11   Ionomers

If polythenes are made with copolymerized acid groups, they may be cross-linked with zinc or sodium ions. The resulting resin marketed as Surlyn A displays excellent sealing properties, even when contaminated with fats and oils. It has low temperature resistance and hot-tack strength. Similarly ethylene-acrylic acid copolymers (primacore) are claimed to give high strength seals and good adhesion to other substrates [6].

12.2.12 Copolymers

When polythene resins are being manufactured, it is possible to mix other monomers with ethylene so that these are incorporated into the polymer molecules. These inclusions alter the characteristics of the polythene. Vinyl acetate is commonly used and the resulting ethylene vinyl acetate (EVA) copolymers display better sealing properties than modifie polythene. Butyl acetate is incorporated with similar effects [9].

12.2.13 Aluminium foil

Aluminium foil is define as a solid sheet section rolled to a thickness less than 0.006 of an inch. Aluminium has excellent properties such as reflect vity, emissivity, thermal conductivity, light weight, corrosion resistance, workability, grease and oil resistance, is tastelessness and odourless, heat and flam resistance, opacity, and non-toxicity. Aluminium foil free from defects is a perfect moisture and oxygen barrier. In applications where good moisture and oxygen barrier properties are important, aluminium foil is always combined with heat sealing media such as polythene or PP. It is the cheapest material to use for the properties obtained. Foils of thickness 8 to 40 μ are generally used in food packaging. As foil is soft and susceptible for creasing, it is generally used as an inner layer.

12.3 Packaging requirements for fish products

12.3.1 Packaging of fresh fish

Fresh fis is one of the most perishable of all foods. More than 20% of the fresh fis caught in many tropical and subtropical areas is wasted. Fish after catch will remain fresh only for a limited period of 4 to 6 hours, depending on the environmental conditions and the intrinsic nature of the fish Chilling by mixing fis with ice is the cheapest and most efficien method of minimizing such wastage. Fish sold immediately in local markets may not need any special packaging. However, a proper packaging to ensure better shelf-life becomes essential when it has to be transported to distant localities. A suitable package for fresh fis should have the following properties:

- provide a barrier against oxygen to reduce fat oxidation;
- keep the fis moist and prevent dehydration;
- retard chemical and bacterial spoilage;
- prevent permeation of external odours.

12.3.2 For bulk packaging

The container should have the following criteria:

- be sturdy enough to withstand the rigors of transit and travel by different modes;
- be of light weight, hygienic, and easily cleanable;
- possess good insulation properties;
- have good barrier properties;
- be returnable or non-returnable on economic considerations.

In some Asian countries, baskets made of split bamboo and similar plant materials are traditionally used for packing fresh iced fish After packing, the outsides are wrapped in gunny and sewed. However, they do not possess adequate mechanical strength and become deformed under stacking. The porous surface of these containers tends to absorb water and accumulate slime, creating an ideal breeding ground for spoilage bacteria, which can contaminate the fis held in them. Used tea chests provided with 2.5 cm thick foamed PS (in polythene sleeving) slabs have been found extremely beneficia for transport of fis over long distances of up to 60 hours duration. Materials such as aluminium, steel, and fibr glass are also used in the construction of insulated containers. A recent development is an insulated corrugated plastic container, which is the lightest of all packages available for iced fis transport. It lasts for fi e trips and being of collapsible design and light weight, return of empty containers is very easy. For cycle hawkers, U-shaped boxes (100 kg capacity) made of HM-HDPE are found ideal. Modern insulated containers are made of HDPE or PP with polyurethane insulation sandwiched between the inner and outer walls of the double walled containers. They are durable and in normal use have a life span of over fi e years [9].

12.3.3   Modified atmosphere packaging (MAP)

Fresh fis is highly susceptible to spoilage due to post-mortem autolysis and microbial growth. The high ambient temperature favours rapid growth of micro-organisms. Presently, ice and mechanical refrigeration are the most common means of retarding microbial and biochemical spoilage in freshly caught seafood during distribution and marketing. However, as ice melts it tends to contaminate fis accelerating spoilage and reduces shelf-life. MAP technology has been developed as a supplement to ice or mechanical refrigeration, to reduce the losses and extend the storage life of fresh seafood products. In MAP, air is replaced with different gas mixtures to regulate microbial activity and/or retard discolouration of the products. The gases normally employed are carbon dioxide, nitrogen, and oxygen. It is primarily the enrichment of carbon dioxide in the storage atmosphere as a means of controlling microbial growth, which results shelf-life extension. Carbon dioxide lowers the intra- and extracellular pH of tissues and possibly that of micro-organisms. Furthermore, it may affect the membrane potential of micro-organisms and influenc the equilibrium of decarboxylating enzymes of micro-organisms. The composition of the gas mixtures used for MAP of fresh fis varies, depending upon whether the fis in the package is lean or oily. For lean fish a ratio of 30% oxygen, 40% carbon dioxide, and 30% nitrogen is recommended. Higher values of carbon dioxide are used for fatty and oily fis with a comparable reduction in level of oxygen in the mixture leading to 40 to 60% nitrogen. By excluding oxygen, the development of oxidative rancidity in fatty fis is slowed. On the other hand, oxygen can inhibit the growth of strictly anaerobic bacteria such as *Clostridium botulinum* [10]. Packaging materials generally employed for this purpose are fl xible film of nylon/surylyn laminates, PVC moulded trays laminated with polythene, and polyester/low-density polythene film etc.

12.3.4   Packaging of frozen fish

Frozen shrimp is the most important item of marine product. Conventionally shrimp is frozen as blocks of 2 kg each in a duplex board carton lined with LDPE and 10 such cartons are packed into 5 or 7 ply corrugated fibr board boxes. The shrimp is frozen with adequate glaze water and the frozen block is once again glazed in ice-cold water before fina packing.

As such, the principal considerations in packaging requirements are adequate strength to withstand handling stress and strain, and resistance to moisture. There is a great demand for shrimp in individually quick frozen (IQF) form. IQF shrimp is a value added product, as it acquires higher unit value compared to block frozen shrimp. The packaging requirements of IQF shrimp vary considerably from those of block frozen shrimp. Greater demand for IQF shrimp is in consumer packs and not in bulk or institutional packs. In the case of block frozen shrimp, it is frozen with glaze and the block is once again glazed, so the risk of moisture loss or oxidative reaction leading to fl vour changes are minimal. However, in an IQF pack, each shrimp is remaining as a discrete piece and it is vulnerable to several risks [1]. Some of the essential requirements for packaging material for IQF shrimp are:

1) low water vapour transmission rate to reduce the risk of dehydration;
2) low oxygen/gas permeability to reduce the risk of oxidation;
3) fl xibility to fi the contours of the food;
4) resistance to puncture, brittleness, and deterioration at low temperature.

In general, the packaging system followed for IQF shrimp is to weigh the product into plastic fil pouches (monofilm co-extruded film or laminated pouches) of a capacity varying from 500 g to 4 kg per pack. The unit pouches may be printed or not and may be provided with unit/intermediate cartons or directly packed in master cartons. The unit/intermediate cartons are made of duplex or 3 ply corrugated fibreboard For IQF packaging, there are several limitations in the use of wax coating on duplex board and hence the preference for corrugated fibreboard However, if a duplex carton is used, in view of the relatively poor quality, it will be desirable to use laminated plastic fil inside the carton as well as to the surface to improve the functional properties as well as aesthetic value. The most functionally effective fil has been identifie as 10 μ biaxially oriented PP (BOPP). One major functional requirement of shipping container/transit package for IQF shrimp is high compression strength to bear weight without damage to the product. It is very important that IQF shrimp should not be subjected to undue pressure during transit and storage. The stack weight should not increase pressure on the product in the cartons in the lower layers. This can be achieved only if master cartons do not yield to pressure and transfer it to the product inside. Compression strength of 500 kg is the minimum recommended specification which might give reasonable safety to the product. Cartons made of 5 or 7 ply corrugated fibr board satisfying the above requirements can be safely used [11].

12.3.5 Packaging of surimi

Surimi is the Japanese term for mechanically deboned fis mince from white-fleshe fis that has been washed, refined and mixed with cryoprotectants for good frozen shelf-life. Surimi needs to be preserved frozen until used for processing into value added products such as fabricated foods, shrimp, and crab analogues and a variety of other products. For this purpose, surimi is generally frozen as rectangular blocks. In order to prevent probable deterioration during storage, such as oxidative rancidity and desiccation, care has to be taken to ensure that the frozen block does not contain any voids and that the packaging materials used have low water vapour permeability and low permeability to gases and odours. The packaging materials employed should be sufficientl strong and durable to withstand stress during handling, storage, and distribution. Packaging employed for block frozen shrimp are considered safe for surimi [10].

12.3.6   Battered and breaded products

This forms an important class of value added products in convenience form. A number of value added marine products, both for export and internal markets, can be prepared from shrimp, squid, cuttle fish certain species of fish and minced meat from low priced fish The changes taking place during frozen storage of the value added products are desiccation, discoloration, and development of rancidity, etc. Application of proper packaging prevents/retards these changes and enhances shelf-life. Conventional packaging materials such as fl xible plastic film alone are not suitable for these products, as they provide little mechanical protection to the products and as a result the products become damaged during handling and transportation. Hence, thermoformed containers are commonly used for this purpose. The thermoformed trays produced from food grade materials are suitable for the packaging of value added fisher products. Trays made of PVC, high impact PS (HIP), and HDPE are unaffected by low temperature and provide protection against desiccation and oxidation during storage.

12.3.7   Packaging of dried fishery products

The special functions required for a suitable packaging material for dried fis products are inertness, leak proofness, impermeability to oxygen and moisture, less transparency, and resistance to mechanical abrasion and puncture. The bulk packaging materials commonly used in the tropics are waxed corrugated cartons, deal wood or plywood boxes, bamboo baskets or gunny bags, dried palmirah or coconut palm leaves, and multiwall paper sacks. Among the different packaging materials studied, HDPE woven gusseted bags laminated with 100 gauge LDPE are found suitable for dried fis packaging. From the hygienic point of view, HDPE is impervious to microbial and insect attack. The commonly used packaging materials for consumer packs of dry fis are LDPE. These materials are cheap, readily available, and have good tearing and bursting strength. Disadvantages are high water vapour and gas transmission rate, and being prone to puncture or damage from sharp spines. In recent years, pouches made of polyester laminated with polythene are commonly used for consumer packs [12].

12.3.8   Packaging of canned fish

A suitable canned fis package should be hermetically sealable, thermally conductive, inexpensive, and should not affect the odour, fl vour, texture, colour, and food value of the contents. Sulphur resistant lacquered cans are generally used for fis products. Common materials used for manufacturing containers for fis products worldwide are tinplate, aluminium, and tin free steel (TFS). Over the years, several improvements have been brought about in can making technology, including the can material. Many of these innovations are the results of the search for can materials that are free of the major defects encountered with tinplate cans such as corrosion and mechanical defects. Cans made of tin-free steel, high tin fille can, and light tin coated steel have been experimented with, but the best promising alternative to tinplate has been considered as aluminium modifie by alloying with magnesium and manganese. Aluminium offers several advantages over tinplate, particularly owing to its light weight, corrosion resistance, easiness to open, and recyclability of metal, etc. The other developments include the introduction of drawn and wall ironed, drawn and redrawn TFS, and welded side seam cans. Another new material, Litewel-N (LTW-N), is being marketed by a Japanese company for use in food and beverage cans [1].

12.3.9   Ready to serve fish products in retortable pouches

Retortable fl xible containers are laminate structures that are thermally processed, such as a can. They are shelf stable and have the convenience of frozen boil-in-the-bag products. The material for fl xible containers must provide superior barrier properties for a long shelf-life, seal integrity, toughness, and puncture resistance and must also withstand the rigors of thermal processing. Retortable fl xible containers may be retort pouches or semi-rigid containers.

The most common form of pouch consists of a 3 ply laminated material. Generally, it is polyester/aluminium foil/cast PP. The outer polyester fil is 12 μ thick. It serves to protect the foil and to provide the laminate with strength and abrasion resistance. The core of aluminium foil is used to give the laminate the necessary water, gas, odour, and light barrier properties. The foil thickness is normally 12 μ, although 7-, 9-, and 15-μ foils are used. The primary function of the PP inner ply is to provide the strong heat seals and good product resistance required in the retort pouch. The layer also helps to protect the foil and contributes to overall pack strength. The three layers of the retortable material are combined by adhesive lamination. Advantages of these retort pouch processed products are that they need not be refrigerated and can be easily opened and served. Generally, processing times can be reduced by as much as 50% as compared to a similarly sized can, jar, or other cylindrical container. There are additional advantages that include reduced shipping costs and storage space for the empty containers. The pouch also has good shelf appeal and a growing acceptance by consumers.

Retort pouches with aluminium foil have the disadvantage of the product not being seen by the consumers before opening. In order to overcome this problem, see-through pouches are being manufactured using laminates of polyester, PP with silicon dioxide, or aluminium oxide coatings, which not only give see-through properties, but also have very good barrier properties. Work carried out at the Central Institute of Fisheries Technology (CIFT) has revealed that fis products packed and processed in these see-through pouches have a shelf-life of more than 18 months at ambient storage temperatures. Now, both opaque and see-through pouches are easily available as they are being manufactured by many industries and are not expensive [13].

12.3.10   Fish sausage

Fish sausage is a product identical to the popular pork sausage. Surimi is the base material, which is homogenized after mixing with several other ingredients. The homogenized mass is stuffed into synthetic casings such as Ryphan (Rubber hydrochloride) or Kurehalon (Vinylidene chloride). The casing is closed using metal rings after which it is heated in water at 85 to 90°C and then slowly cooled. After drying the surface, the sausage is wrapped in cellophane laminated with polythene. Fish sausage is kept at refrigerator temperatures for retail; however, when prolonged storage is needed it is better kept frozen. Duplex cartons lined with a plastic fil are ideal for short-term storage, but when stored frozen, packaging suggested for block frozen shrimp is considered suitable [14].

12.3.11   Accelerated freeze dried (AFD) products

Application of the technique of freeze drying in fis preservation is becoming very popular in spite of the high cost of production because of several other advantages associated

with the products. These are practically devoid of moisture, its percentage generally less than 2. The products are very fragile and can easily undergo chemical reactions with air leading to oxidation, deterioration of colour, and absorption of water. They are generally packed under an inert gas to exclude air and oxygen. Hence, the main requirements in the packaging employed are low oxygen and water vapour transmission to protect the product from rancidity and absorption of moisture and sufficien mechanical strength to protect from shock. Paper/aluminium foil/polythene laminates or metallized polyester polythene laminated pouches or metal cans are recommended for these products [15].

12.3.12   Fish pickles

Fish pickle is a value added item prepared from low cost fish/shel fis meat, and other ingredients such ginger, chilli, and acetic acid, etc. Conventionally, glass bottles are used that offer properties such as inertness, non-toxicity, durability, non-permeability to gases, and moisture. However, they are heavy, prone to breakage, voluminous, and expensive. New fl xible packaging materials developed for fis pickle is based on plain polyester laminated with LDPE-HDPE co-extruded fil   or Nylon/Surlyn or LD/BA/Nylon/BA/Primacore. These are inert and can be attractively fabricated as stand-up packs and can be printed on the reverse side of the polyester fil   [16].

12.3.13   Fish soup powder

Fish soup powder is a specialty product containing partially hydrolyzed fish protein, carbohydrates, fat, and several other seasonings including salt. The product is hygroscopic and hence the selection of the package assumes great significance Appropriate package developed for such products are 12 μ plain polyester laminated with LDPE-HDPE co-extruded fil   or 90 to 100 μ LD/BA/Nylon/BA/Primacore multilayer fil   [17].

12.3.14   Shark fin rays

Dried shark fi is a popular product on the international market. Significan value addition is possible if the rays from shark fin are extracted and exported in place of shark fins Moisture resistant packaging having good puncture resistance and sufficien mechanical strength to withstand the hazards of transportation are the major requirements in the packaging for shark fi rays. Polyester/polythene laminates or Nylon based co-extruded film are appropriate [16]. Traditionally dried shark fin are packed in bulk in jute sacks. The improved bulk pack consists of a HDPE woven sack or a PP woven sack.

12.3.15   Chitin/chitosan

Until recently, positing is a very serious problem in its disposal and shrimp waste has now become a very valuable commodity because of its use in the manufacture of chitin and chitosan. The packaging should protect the product against moisture as well as microbial and insect attacks. The packaging developed for chitin/chitosan and now extensively being used by the industry is a HDPE woven gusseted bag laminated with 100 gauge LDPE [16]. Apart from these, application of chitinous products in foods and pharmaceuticals as well as processing aids has received considerable attention in recent years as exotic synthetic

compounds are losing their appeal. Shahidi *et al*. [18] reported a detailed review on the application of chitin and chitosan in food.

12.4 Safety aspects of packaging materials

Many types of packaging materials are being used in the fis processing industry. There is widespread concern on the increasing presence of packaging materials in the waste stream and their effect on the environment in industrialized nations. Environmental legislations concerning packaging are being enacted aimed at their reduction at source or to facilitate their recycling or reuse, through incentives, penalties, voluntary, and mandatory restraints. Hazardous substances migrating or permeating from the packaging materials and their components into the foodstuffs coming in contact with them and affecting the health and safety of the consumer are also of equal concern. Plastics are increasingly used as packaging material for fis products and migration of leftover residual monomers and additives may impair the quality of packed products. Hazardous metals and volatiles in printing inks and can solders, if permeating beyond acceptable limits, can endanger the health of consumers. There are laws regulating the limits of these hazardous substances in packaging materials and ingredients in food products and there are complete bans on certain packaging materials in some countries. These laws and restrictions vary geographically and the exporters of marine products have to be aware of these, so that these products are not rejected or held up at destination markets due to unacceptable packaging.

When selecting an appropriate packaging system for a food product, a number of criteria must be considered. Foremost is the stability of the food product itself as food components such as protein, lipid, and certain vitamins may undergo detrimental changes due to variation in water activity of the product. The stability of the product will thus be a function of its chemical, biochemical, and physical nature and will be influence markedly by the permeability or barrier properties of the package. Secondly, environmental factors, such as temperature, relative humidity, oxygen tension, and light intensity, to which the product/package system is exposed during distribution and storage, must also be considered when evaluating the barrier properties required for the package. Lastly, the nature and composition of the specifi packaging material and its potential effect on the intrinsic quality and safety of the packaged food as a consequence of the migration of components from the packaging material into the food should also be considered.

Plastics and plastic based materials are increasingly used in the fis industry either as containers/crates for storage of raw materials or processed fis at factories, or fina packaging in semi-rigid and other fl xible forms. Some of the plastics commonly used for fis packaging are LDPE, HDPE, LLDPE, HM-HDPE, PP, polyester, nylon, PS, ethylene acrylic acid (EAA), and polyacrylonitrile. All plastics, apart from the basic polymers, contain several non-polymeric components, either inherent or deliberately added to plastics, which are classifie into three categories [19]:

1) Polymerization residues (residual monomers, catalyst remnants, polymerization solvent, etc.);
2) Processing aids (plasticizers, stabilizers, antioxidants, slip agents, lubricants, antistatic agents, etc.);
3) End-use additives (antioxidants, brighteners, blowing agents, mould release agents, colourants, UV stabilizers, etc.).

Among the above, the firs type of compounds is unavoidable, whereas those of the other two types are deliberately added to the polymer either during manufacture or subsequently to achieve the desired end properties of the finishe plastic material. Polymers themselves being of high molecular weight are inert and have limited solubility in aqueous and fatty systems. However, the non-polymeric components may leach out from plastics to foods whenever direct contact occurs between food and plastics, thereby contaminating the food product with the consequent risk of toxic hazard to the consumer [20–22]. The awareness in this matter has led the national and international regulatory authorities in the reckoning of guidelines for proper use of plastics for food packaging application. Such guidelines are necessary to restrict the indiscriminate use or abuse of plastics in food packaging.

Different countries such as India, US, UK, Europe, and Japan have laid down specifi cations and codes of manufacture for the safe use of plastics for food contact applications [23–35]. This relates principally to the use of various ingredients, additives, and other processing aids used by the manufacturer in the formulations of plastics composition. These recommendations are based on the existing toxicological data. In this regard, positive list of constituents, which are generally regarded as safe (GRAS) and specification for safe use of plastics commonly used in food packaging, have been laid down. The residual monomer and heavy metal contents in different plastics specifie by different countries are presented in Table 12.1. Limits of heavy metals in colours used in plastic manufacture are as follows: lead (0.01%), arsenic (0.005%), mercury (0.005%), cadmium (0.20%), selenium (0.20%), and barium (0.01%) [36]. Other regulations on food packaging materials comprise of regulations for adjuvants (antioxidants, colourants, and plasticizers, etc.) used in food packaging materials. Only cited materials within given limits, such as the amount of the adjuvant which can be used and the kinds of plastic in which it can be used, are described in Table 12.2 [37].

The third aspect is the extractive limits for the fina food contact article. Here, the limitations would thus include the contributions from all the adjuvants and processing aids used in making the food contact packaging material. These regulations spell out the time/temperature/solvent conditions for the short-term extraction experiments (migration tests) used to test compliance. Migration tests for adjuvant transfer into foods should be conducted with each type of food in a given package under normal conditions of use for an expected contact time. However, apart

Table 12.1 Limits of monomer and heavy metals in plastics. Adapted from Anon [36]

Country	Monomer	Heavy metals
BIS-India	VCM in PVC:1 ppm In food migration: 10 ppb, Styrene in polystyrene: 2000 ppm	Lead: 1 ppm Others: 0.01 ppm in PVC
EEC-Europe	VCM in PVC: 1 ppm	Nil
EPF-UK	VCM in PVC: 1 ppm Styrene in PS: 5000 ppm	Nil
Japan	VCM in PVC: 1 ppm	Lead, cadmium, and barium: 100 ppm each in PVDC
	Volatile component in polystyrene: 5000 ppm Vinylidine chloride in PVDC: 6 ppm Caprolactum in Nylon: 15 ppm	Antimony: 0.05 ppm in PET Germanium: 0.1 ppm in PET
FDA-US	VCM not specified styrene in PS: 10000 ppm Acrylonitrile in ABS plastics: 11 ppm	Nil

Abbreviations: VCM, vinylchloride monomer; PVC, polyvinyl chloride; PVDC, polyvinyl idenechloride; PET, polyester.

Table 12.2 Permitted additives in finished packaging materials. Adapted with permission from Gopal & Ravi Shankar [37]

Additive type	LDPE/LLDPE	HDPE	PVC	PS	IONO-MER	EAA
Polymerization residues (%)						
Calcium, aluminium, silicon, and titanium (%)	0.2	0.2	–	–	0.2	0.5
Chromium (ppm)	–	50	–	–	–	–
Emulsifying agents (%)	0.3	0.3	0.3	–	–	–
Catalyst (%)	0.2	0.2	0.25	0.2	–	–
Lubricants (%)	2.0	2.0	–	–	2.0	1.0
Stearyl ethylene diamine (%)	–	–	–	–	–	0.2
Fatty acid amides (%)	0.2	0.2	0.3	0.3	–	–
Microcrystalline waxes, paraffin or oil (%)	0.1	–	–	2.0	–	–
Octoates, oleate, palmitate & stearate of zinc (%)	2.0	–	–	0.1	–	–
Poly (1,2 propylene glycol) (%)	0.1	–	–	–	–	–
Phthalates of monovalent alcohols (%)	0.2	–	3.0	–	–	–
Polyethylene glycol (%)	–	–	–	0.1	–	–
Stearyl erucamide (%)	–	–	–	–	–	0.2
N, N-bis-stearyl/palmityl ethylene diamine (%)	1.0	1.0	–	–	–	–
Fatty alcohols (%)	–	–	3.0	–	–	–
Antioxidants (%)						
4,4-thio-bis (6-t-butyl-n-cresol) (%)	1.5	1.5	–	–	2.0	1.5
4,4-butylidine-bis (6-tert-butyl-n-cresol) (%)	0.25	0.25	–	–	–	–
1,3,5-trimethyl-2, 4,6-tris (3,5-ditert butyl-4-hydroxy-benzyl) benzene (%)	0.3	0.3	–	–	–	–
2,4, dinonyl phenyl,di(4-mononylphenyl) phosphite (%)	0.5	0.5	–	–	–	–
2,2-methylene-bis-6- (1 methyl-cyclohexyl) p-cresol (%)	0.2	0.2	–	–	–	–
Antistatic agent (%)	0.5	0.5	–	–	0.5	0.5
Tri-iso-propanolamine (%)	0.5	–	–	–	–	–
N-N-bis (2-hydroxyethyl) alkyl amines (%)	0.3	0.3	–	–	–	–
Cetyl pyridinium chloride (%)	0.4	0.4	–	–	–	–
N, N-bis (Polyhydroxyethyl) alkyl amino	–	0.3	–	–	–	–

Abbreviations: LDPE, low-density polyhene; LLDPE, linear low-density polyhene; HDPE, high-density polyhene; PVC, polyvinyl chloride; PS, polystyrene; EAA, ethylene acrylic acid.

Table 12.3 Global Migration Limits (GML) in various specifications

Standard/country	GML	Reference
BIS (India) BPF (UK) EEC (Europe)	60 mg/kg or 0.1 mg/cm^2 for all polymers, for which specifications are available.	[23,23,31,33,39]
FDA (US)	50 mg/lit or 0.75 mg/cm^2 for resinous and polymeric coatings. 21–197 mg/in^2 for rubber articles. 0.15 mg/in^2 (water) for phenol formaldehyde moulded article. 0.02–0.5 mg/in^2 for polyesters (depending on use and conditions). 0.2–2.5 wt. Percent for various nylons depends on the extractive solvent.	[35]
JIS (Japan)	150 mg/lit for PE and PP. 30 mg/lit for containers to be used at >100 °C. 15–30 mg/lit for Nylon.	[32]

from being economically prohibitive, this type of evaluation with actual foods is analytically difficul because of their complex nature. Foodstuffs vary in composition from place to place and more importantly they are unstable and decompose fairly rapidly. Furthermore, the duration involved makes long-term tests with foodstuffs impractical. Food simulating liquids such as water, ethanol, acetic acid, and heptane have been recommended to be used in place of actual foodstuffs [21].

Foods are divided into several types to determine the overall migration residue. Methods for determination of migration residues depends on the type of food, simulating solvents, time, and temperature [35,38,39]. Limits for migration residues are given in Table 12.3. Tinplate has been used to make food containers for over 160 years. Many cases of food poisoning occurred, due to ingestion of excessive amounts of metal. Levels of tin in food up to 250 ppm are generally tolerated by regulatory authorities and higher levels in food cause gastrointestinal disturbances. The sideseams of three-piece cans are soldered with a lead/tin (98:2) solder, resulting in some lead being taken up by the food, depending on the amount of solder exposed to the food and the acidity of the food. Some lead contamination may also originate from the tin coating, in which it may be present as an impurity. Regulatory limits for lead in almost all countries are now 2 ppm in canned foods. The newer welded cans have eliminated solder altogether and reduced the lead intake from canned foods to about one tenth.

Aluminium has a long history of safe usage in connection with food and food packaging and is recognized as GRAS material by the US Food and Drug administration (FDA). The discovery of aluminium in senile plaques of patients with Alzheimer's disease has led to the suggestion that chronic exposure to low levels of aluminium in water and foods may be implicated in the etiology of this form of dementia. The limit is 1 mg/kg body weight per day recommended by World Health Organization (WHO) [40].

12.5 Conclusions

Considering the importance of seafoods, it is essential to devote attention to produce and market good-quality seafood products for both export and internal markets. Even though

strict quality control practices are implemented for ensuring food safety for the export trade, there are no proper guidelines with regard to quality and safety of packaging materials used. It is highly essential that appropriate packaging material is used for a specifi fis product and testing of packaging materials should be made mandatory for parameters, such as overall and specifi migration of residual monomers and other toxic components.

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13 Fish mince: cryostabilization and product formulation

Chong M. Lee

13.1 Introduction

There are still many fis species, by-catches and processing by-products worldwide remaining under- or unutilized. The unutilized stocks can be converted into refine and cryostabilized mince blocks for further use in the development of various value-added seafood products with innovative formulation strategies. The trend of developing formulated mince-based seafood products is growing, as evidenced at various seafood trade shows. This chapter focuses on identifying the source of commercially viable fis mince, cryostabilization of fis mince, lipid oxidation, and its control of high omega-3 fatty acid mince, appropriate formulation strategies based on their physical characteristics and types of products to be developed, feasibility of manufacturing surimi-mince blend products, the role of ingredients and processing methods in texture and fl vour modifications and frozen storability.

13.2 Background information

13.2.1 Rationale for the development of fish mince technology

With ever increasing pressure on the supply of traditional fis species from the ground fis stock diminishing in recent years, effective utilization of non-traditional fis species as well as processed by-products becomes vital to the survival of today's seafood industry worldwide. Currently, there are several species in the Northwest Atlantic Ocean that are identifie as not overfished though seasonal. They include mackerel, herring, and red hake [1]. In addition, pink and chum salmons in the Northeast Pacifi Ocean are not being optimally utilized, although they are available in abundance. Along with such non-traditional and less optimally utilized species, there is also an oversupply of processed by-products, such as fis frames and trimmings from cod, Alaska pollock, catfish flounde , and other ground fis from the filletin operation. For example, the volume of pollock processing by-products was estimated to be 550,000 metric tonnes in 2008 [2]. The breakdown of by-product components was 33% frames, 32% viscera, 26% heads, and 9% skin [3].

The recent development of highly acceptable mince-based products (cheddar cheese-fl voured mackerel nuggets and scampi sauce-fl voured herring nuggets) [4] led to the idea of separating refine meat and freeze stabilizing the mechanically recovered mince without washing to produce a variety of value-added convenient seafood products, including products with a health concept such as high omega-3 fatty acid products. The significanc of this idea is to develop a new seafood base material and a manufacturing industry in a manner similar to the development of surimi and related industry. The potential advantages of the refinin and cryostabilization process include no washing, hence no high biochemical oxygen demand (BOD) effluent high yield, retention of nutrients, inventory control of frozen stable intermediate product to be used in formulated seafood products, and the process being inclusive of various fis species, by-catches, and processing by-products.

13.2.2 Source of fish mince

Currently, fis mince blocks are being produced primarily from Alaska pollock (*Theragra chalcogramma*), Pacifi (*Merluccius productus*), Atlantic whiting (silver hake, *Merluccius bilinearis*), and Argentine hake (*Merluccius hubbsi)* for the manufacture of economic grade fis sticks. However, no attempt has been made to manufacture mince-based products from the dark-fleshe fis species. Various potential sources of fis mince include headed and gutted (H&G): pink salmon and Alaska pollock; trimmings and frames (species of volume production): Alaska pollock, cod, catfish salmon, tilapia, and whiting; and under-utilized species: mackerel and herring. For frame mince, no viable market exists other than manufacturing a second grade surimi. An earlier work [5] showed that the mince from cod frame can be cryostabilized with some loss of functionality. However, no attempt was made in this study to develop formulated mince-based products.

Lack of market appeal for traditional forms of products (e.g. batter-breaded) from the dark fles fis species as well as the limited use of frame mince has led to designing newly engineered products from fis mince with an appropriate technology and a novel formulation strategy (Fig. 13.1). In recent years, there have been many mince-based formulated products developed from various fis species, notably salmon and tuna. The products include nugget, patty, sausage, frankfurter, and ham. The recent development of well received formulated tuna nuggets has opened the door for new mince products to be developed from other species. Our work [4] revealed that a highly acceptable mince-based product with a score of 7 to 8 out of 9 (9 being excellent) can be produced from the light fles of mackerel when
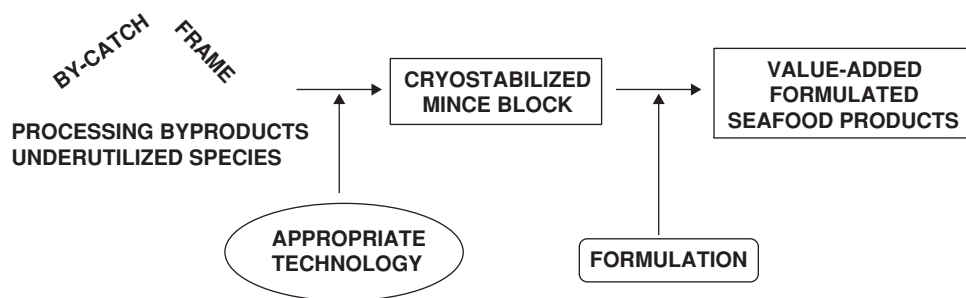
Fig. 13.1 Development of fish mince-based formulated seafood products from various underutilized resources through appropriate technology and formulation strategy. Adapted from Lee [6].

appropriately formulated. Developing new mince-based products from different fis species and filletin by-products requires a comprehensive analysis of species' characteristics with respect to the physical and sensory properties of fis mince. They include texture-forming properties, fl vour and colour characteristics, frozen storability of mince block, and freeze-thaw stability of cooked mince. Once a suitable product form for the mince from each species is identified appropriate formulation is designed according to its product-forming and organoleptic characteristics. There has been some work reported on fis mince in relation to product development and chemical degradation, but no study has been conducted to develop an effective method to cryostabilize unwashed fis mince and to characterize fis mince with respect to physical and sensory properties.

13.3   Manufacture of fish mince and cryostabilization

The prerequisite to a successful development of fis mince-based products is the availability of raw material in stable supply with consistent quality. There are two steps to be taken in manufacturing fis mince-based products. They are cryostabilization of fis mince and formulation of products to be developed.

13.3.1   Manufacture of fish mince

In fis mince preparation and cryostabilization, three important elements should be taken into account. These include effective mechanical removal of dark fles from fis species such as mackerel and herring, minimization of blood and viscera inclusion in the finishe mince, and cryostabilization of mechanically separated mince. A general fl w diagram of fis mince production is given in Fig. 13.2. The important points in designing the mechanical mince production process are: the process must be species specific H&G with thoracic bone removed for the gadoid fis species (e.g. cod); H&G for the mid-water species of small size (e.g. sea bream); fille for pelagic fis species (e.g. mackerel, herring); and the use of a 3 mm drum.

FILLET   H & G
                         FRAME
          REMOVAL
             OF
(DARK FLESH)   (THORACIC BONE)

MEAT-BONE SEPARATOR

MINCE

(REFINING)

(CRYOSTABILIZATION)

CRYOSTABILIZED MINCE BLOCK
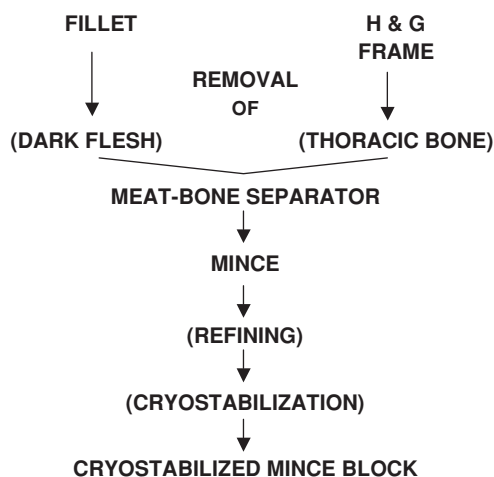
Fig. 13.2   Flow diagram of fish mince production.

13.3.1.1 *Manufacture of fish mince from ground white fish*

The common problem with conventionally prepared fis mince from frames or H&G ground fis (typically lean and white) is inclusion of blood and viscera, which results in poor colour and fl vour and texture changes during frozen storage. A simple solution to this problem is to remove the thoracic cavity which harbours the blood and viscera. The thoracic cavity from cod frame can be removed mechanically by a stamping-type time-controlled cutter. Distribution and yield of different parts of cod frame are given in Fig. 13.3. Viable sources of fis frames that yield a good portion of mince are ground fis (cod, haddock and pollock), salmon, catfish and tilapia. Washing is an effective means for removing blood to whiten the mince, but it also removes large amounts of fl vour and nutrients that are characteristic to each species. However, washing improves frozen stability of fis mince, especially when cryoprotectants are added. When cod frame mince with the thoracic cavity removed was cryostabilized with 8% liquid polyol and 0.2% sodium tripolyposphate (STPP), stored frozen for 2 months, and washed (3 volume of water, 2 washings), it retained 63% of the functionality (gel-forming ability) of the mince that had been washed, cryostabilized, and stored frozen for 2 months [5,6]. Polyol (ICI America, Wilmington, DE) was composed of 58.5% sorbitol, 11.5% mannitol, and the remainder with water. However, when fis mince was prepared with two washings from frames where the thoracic cavity remained, it suffered considerable
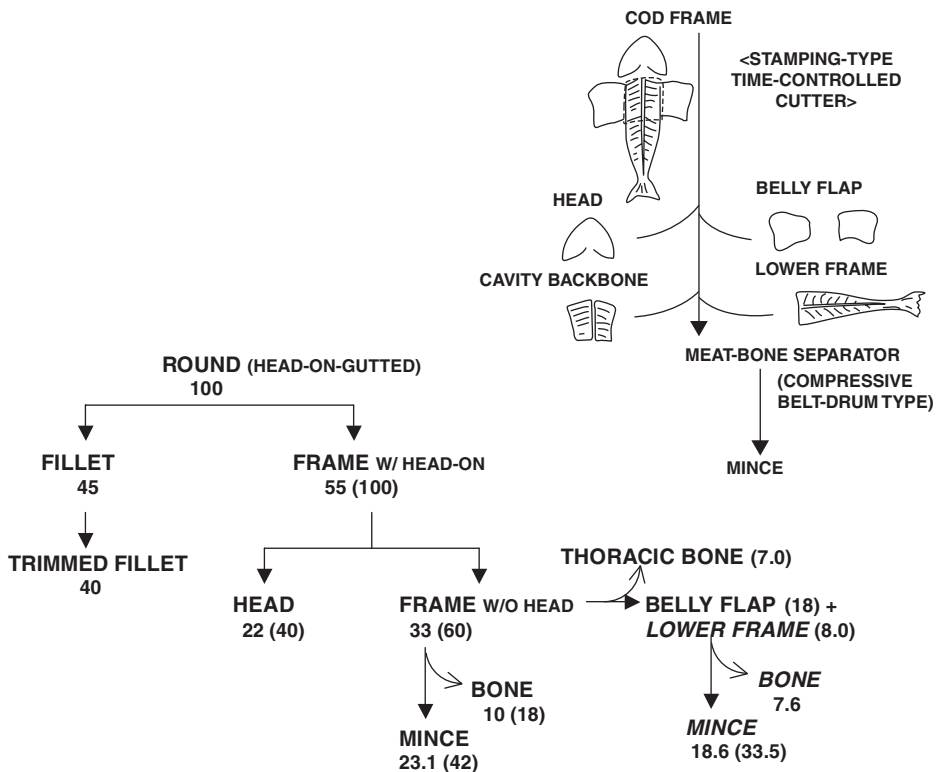
Fig. 13.3 Distribution and yield of different parts after mechanical deboning of Atlantic cod frame (based on 45% yield of fillet by manual cutting of an average size of 4–5 lbs head-on-gutted cod). Adapted from Lee [5].

loss of functionality by 44% after 2 months of frozen storage. Whiteness, as indicated by the L^* value, clearly reflecte the effects of removal of thoracic cavity, washing, and cryostabilization, where it was interesting to note that cryostabilization improved whiteness. It is believed that the colour changes occur as muscle fibre undergo considerable freeze shrinkage in the absence of cryoprotectants.

13.3.1.2 *Manufacture of fish mince from pelagic dark fish*

As for the mince production from the dark fles fis such as mackerel, removal of dark fles from mackerel fille can be done by an appropriate mechanical device, modifie from the skinning machine, which has a capability of deep skinning and carving the dark fles line located along the lateral line (Fig. 13.4). We found that the light meat is fl vourful and appears to be stable against fl vour changes from rancidity. If an effective means of mechanical removal can be devised, a potential market for the mackerel light mince block can be realized in developing a variety of formulated mackerel mince-based seafood products. For herring, on the other hand, the whole fis are fed through a filletin machine, followed by
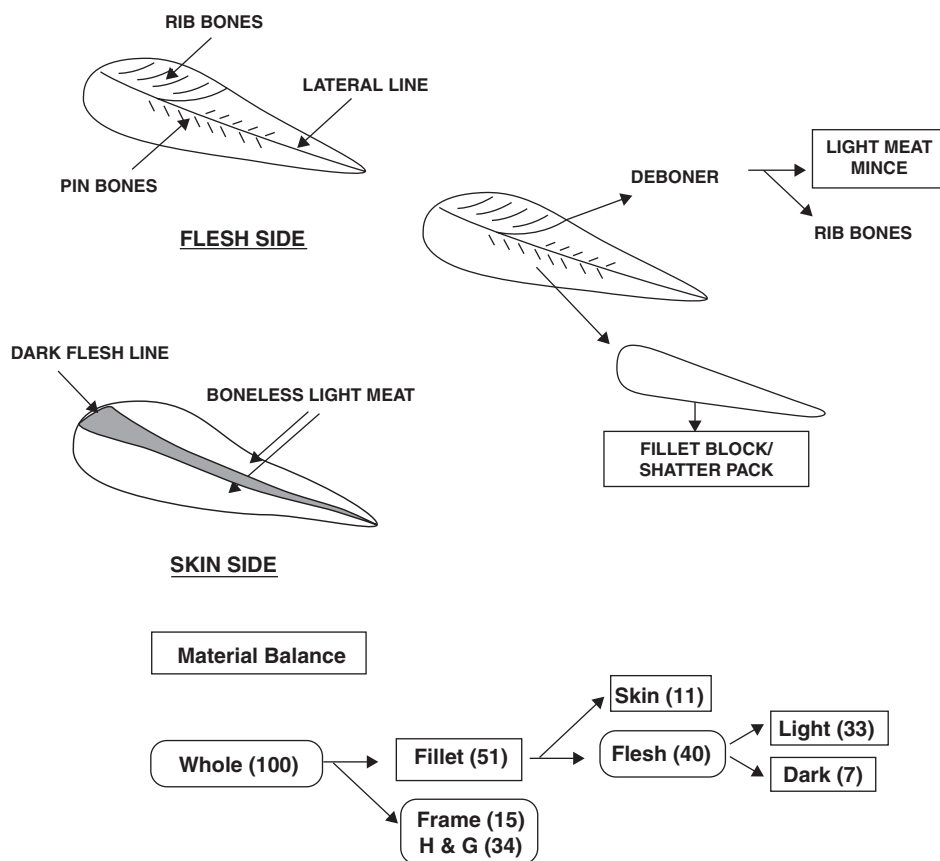
Fig. 13.4 Production and yield of light meat and mince from the skin-off mackerel fillets. Adapted from Lee *et al.* [4].

WHOLE → BAADER FILTER 235 → FILLETS (SKIN-ON)

→ DEBONER W/ 3-MM DRUM → MINCE (41% YIELD)

(SKIN, DARK FLESH)   CRYOPROTECTANT

CRYOSTABILIZATION

FROZEN STABLE MINCE BLOCK
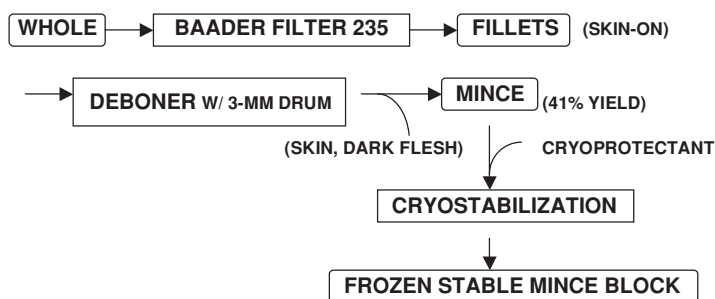
Fig. 13.5 Proposed production of cryostabilized mince block from Atlantic herring and mackerel.

mechanical deboner to produce mince (Fig. 13.5). A high-speed continuous filletin machine (Model 235) for both herring and mackerel is available from Baader Machineries in Germany. A 3 mm drum is recommended for the production of fis mince to remove all scales and bone particles. A drumhole size smaller than 3 mm may destroy the integrity of muscle fibre resulting in the loss of fibrou texture. The temperature of the material must be kept below 10°C for the entire duration of the mechanical deboning process to minimize deteriorative enzymatic as well as oxidative reactions. However, its high levels of polyunsaturated fatty acids (PUFA) combined with mechanical mincing is believed to make oily fis mince prone to oxidative rancidity during frozen storage, leading to the development of fis y odour. Mincing destabilizes the fis tissue due to exposure of tissue lipid to atmospheric oxygen, cellular disruption [7], and subsequent activation of lipoxygenase [8,9], and the release of prooxidant and haemoglobin from erythrocytes [10]. Lipoxygenase present in fis tissue can initiate the oxidation of PUFA to produce volatiles responsible for oxidative odour, namely, "fis y odour" [11]. Thus, controlling lipid oxidation and inhibiting lipoxygenase can reduce fis y odour [12]. Medina *et al*. [8] and Mansur *et al*. [13] found that lipoxygenase was concentrated in the skin tissue. Mechanical deboning of fillet with skin on will, therefore, activate this enzyme.

Following mechanical mincing of herring, the thiobarbituric acid reactive substance (TBARS) values increased, even during the 40-minute period of post-deboning and handling [14]. The rate of TBARS increase in the mince without milk protein concentrate (MPC) (0.0183/min) was found to be almost twice as fast as that of the mince with MPC (0.0095/min). MPC 56 (formerly Alapro 4560) was provided by NZMP Fonterra Ltd. (Santa Rosa, CA). As for fatty acids, the retention of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was improved by the addition of MPC. Such time-dependent changes in TBARS and fatty acids in a relatively short period of time clearly suggest that mincing does affect lipid oxidation in fis tissue, probably through cellular disruption and subsequent activation of lipoxygenase as described previously. These results suggest the enzymatic degradation of fis tissue lipids as being rapid and the importance of immediate treatment of minced fis tissue with an appropriate type of antioxidants. Lipid oxidation starts during mixing and packing, and adding at least 4% MPC has proved effective in slowing down lipid oxidation, even prior to frozen storage [14,15]. Another point that needs to be addressed is incorporation of metal particles from a non-stainless steel knife used in the deboner, which could trigger an iron-catalyzed lipid oxidation, as reported by Lee and Toledo [16]. This is of particular concern to fatty dark fles fis species, and not so much to lean, white fis species.

The quality problems during frozen storage of fis mince are associated with the development of rancidity in fatty fis and texture hardening in lean fish The texture control by added proteins in our earlier study [17] was the basis of our recent work of cryostabilization of herring, mackerel, and red hake mince [4,18].

Pink salmon (*Oncorhynchus gorbuscha*) is the most abundant species of salmon in Alaska and is mostly processed by canning. Reppond and Babbitt [19] investigated the processing of pink salmon into boneless fillet and mince and changes in quality during frozen storage at $-18°C$. Use of minced salmon increased the yield of edible product by one-third. Desirability scores tended to be higher for blocks made from unfrozen fis than for blocks made from previously frozen fis and higher for blocks with 0 or 25% mince than for blocks with 50 or 100% mince. One exception was chewiness, which tended to increase more for blocks made from unfrozen fis than for blocks made from frozen fish Changes in colour and rancidity values were small.

13.3.2   Quality evaluation of fish mince

The evaluation of fis mince quality depends upon fis species from which mince is prepared, and the application where and how the mince will be used. For the routine quality control purpose, one can refer to the US standards for grades of frozen minced fis blocks [20] and the CODEX Standard for quick frozen blocks of minced fis   fles and mixtures of fillet and minced fis   fles [21].

Deleterious quality changes in frozen fis mince are associated with texture hardening or toughening, poor dispersibility (the condition in which the frozen-thawed mince is not easily dispersed when mixed with ingredients), poor functionality (e.g. gelling, formability and water holding ability), discoloration (darkening), lack of fla y texture, and lipid oxidation and fis y odour development. These cause a major problem in developing mince-based seafood products, especially the mince produced from lean, white fis species. Texture changes can be measured following the method designed for raw and cooked fis mince [18]. Lipid oxidation and fis y odour development can be assessed following the methods of Joaquin *et al*. [14].

13.3.3   Cryostabilization of fish mince

The common problem with frozen fis mince is texture hardening and lack of moisture in the finishe products. This results from freeze syneresis accompanied by freeze shrinkage of muscle fibres The water-binding proteins (e.g. soy, egg, and milk proteins) are found to have the ability to keep muscle fibr from shrinking during frozen storage, as reported by Yoon *et al*. [17]. There was a good correlation between water binding properties (physically-bound water, not differential scanning calorimeter (DSC)-measured chemically bound water) and the ability to reduce freeze contraction.

In our earlier studies [18,22], a two-prong approach was made to cryostabilize fatty pelagic and lean demersal fis using a combination of antioxidative proteins and cryoprotective polyols. The fis y odour development and the frozen-induced texture hardening are primary concerns with the fatty pelagic and lean gadoid fis mince, respectively. Mackerel and herring are fatty pelagic fis and present a problem with lipid oxidation, which leads to development of fis y odour and rancid taste. Unlike the lean gadoid fish texture deterioration does not appear to be a problem in the pelagic fish For this reason, antioxidative proteins were examined for pelagic fis mince, while cryoprotectants were evaluated for lean fis mince.

In texture hardening of lean fish calcium ion has been implicated since it may promote the formation of ionic cross-linkages between polypeptide chain [23] or catalyze an acyl-transfer cross-linking reaction of myosin heavy chain by activating a Ca^{2+}-dependent transglutaminase (TGase) [24]. Thus, the removal of calcium ions by calcium chelating hydrocolloid such as alginate [25] may repress fis protein cross-linking formation. The freeze-induced textural hardening and poor dispersibility in red hake mince were controlled by the combined use of 0.2% soy protein concentrate (SPC) – 0.4% alginate – 4% sorbitol – 0.3% STPP, where alginate (Keltone) was supplied by Kelco (San Diego, CA). The dispersibility of fis mince is an important quality requirement for the ease of mixing with other ingredients during formulation. The preventative role of alginate in texture hardening is believed to be related to its ability to chelate calcium ions responsible for cross-linking and interfere with muscle fibr interaction [22].

The calcium content [26] in red hake (29.4 mg/100 g) was found to be significantl greater than in Alaska pollock (16.5 mg/100 g) and Pacifi whiting (8.7 mg/100 g) [27], suggesting that it plays a key role in texture hardening of fis mince since uncooked red hake mince (400 g penetration force) became significantl harder than Alaska pollock mince (150 g).

The total free-sulphydryl (SH) content in the control red hake mince significantl decreased over the 17-week frozen period [22]. This was consistent with the reduction of sodium dodecyl sulphate (SDS)-soluble protein, implying the involvement of the formation of disulphide bond in protein insolubilization of frozen mince. These finding were further supported by the result of SDS-PAGE. After 17 weeks of frozen storage, no change in myosin heavy chain (MHC) band of 0.4% alginate – 4% sorbitol – 0.3% STPP was observed, while a noticeable reduction in MHC of control indicated that MHC in the control underwent polymerization through S–S cross-linking.

Upon screening with a 4 freeze-thaw cycle test, 6 proteins were selected out of 14. They were soy protein isolate (ProFam 648, ADM, Decatur, IL), soy protein concentrate (SPC; Arcon S, ADM), milk protein isolate (TMP-1350, NZMP Fonterra Ltd., Santa Rosa, CA), MPC (MPC 56, formerly Alapro 4560, NZMP Fonterra Ltd.), whey protein concentrate (Alacen 878, NZMP Fonterra Ltd.), and egg white (spray dried, Papettie Hygrade, Elizabeth, NJ). These six were incorporated at 4% into new batches of herring and mackerel mince and subjected to a 6-month frozen storage test at $-18°C$ using the same evaluation parameters. The two best performing and cost-effective proteins, namely, soy (Arcon S) and MPC 56, were selected and subjected to another round of 6-month frozen storage tests using 0, 2, 4, and 6% for determination of the respective optimum level in herring, mackerel, and red hake mince.

The most effective level was 4%, with the minimum level at 2%. The different responses (TBARS and fis y odour) to MPC and SPC could be explained by the characteristic binding between protein and low molecular weight (LMW) reactive substances in the muscle [28].

An interesting findin was that MPC (lower TBARS, fis y odour, and penetration force) was suitable for pelagic fis mince, while SPC was better suited to red hake mince. After a 6-month frozen storage of red hake mince, no fis y odour was detected in both mince prepared with MPC and SPC. However, the differences were observed in hardness (SPC: 6.5, MPC: 8.5, 1 soft – 9 hard) and texture desirability (SPC: 5, MPC: 2, 1 undesirable – 9 very desirable). The texture moderating effect of soy protein was due to its physical interference with muscle fibr interaction such as cross-linking between protein molecules [17]. The addition of proteins with a minimum of 2% controlled the development of fis y odour, a sign of oxidation of low molecular reactive compounds including fatty acids in both red hake and pelagic fish This is believed to be a result of protein binding with such reactive

compounds, making them less prone to oxidative reactions responsible for the development of fis y odour.

Joaquin *et al*. [14] demonstrated that the addition of 4 and 6% MPC to herring mince resulted in a 33 and 50% reduction of TBARS, respectively, at month 4 and lessened the intensity of fis y odour throughout the storage. However, MPC did not protect fatty acids from enzymatic degradation unless it was added immediately after mincing. Volatile analysis using static headspace analyses-gas chromatography-mass spectrometry (SHA-GC-MS) showed that 4% MPC was able to reduce headspace volatiles associated with fis y odour. MPC is the most effective to reduce 4-heptenal, 3-methyl-1-butanol, 2-hexenal, and 1-penten-3-ol, which are known to be potent odorants associated with lipid oxidation.

13.4 Formulation of fish mince-based products in relation to ingredients and sensory quality

Once the source of fis mince is secured, products need to be developed and markets identified In order for the product to stand in the competitive market with a good sensory profil (texture, taste, and colour), one should be mindful about formulation and selection of ingredients. A successful entry into the market requires a sound strategy for product identificatio and formulation. Today, the most identifiabl products may include nugget, sausage-type, spread, and portion. The portion is applied when no formulation is involved. A general production scheme for formulated mince-based products is outlined in Fig. 13.6. Unlike traditional formulated meat products, fis mince would yield products with soft and less meaty texture. This presents a formulation challenge when the textural characteristics similar to those of meat products are desired. In our earlier work on the development of smoked sausage links from Spanish mackerel (*Scomberomorous maculatus*), the use of shortening and soy protein fibr (SPF) with a two-stage comminution resulted in the most desirable texture with adequate firmness chewiness, and juiciness [29]. The two-stage comminution process consisted of initial chopping of SPF, fis mince, ice, salt, corn syrup, and shortening, followed by mixing and grinding of chopped batter with the remaining mince, and salt in a bowl mixer and through a 4.5 mm plate meat grinder, respectively.
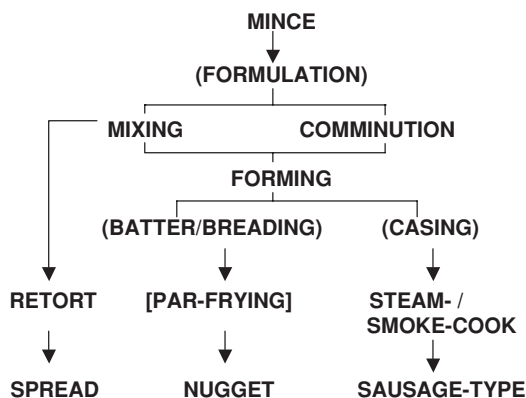
Fig. 13.6 Flow diagram of manufacture of various types of formulated mince-based products.

MINCE INGREDIENTS COMPOSITE

DISPERSION → → THERMAL → STABLE
ACTIVATION PRODUCT

WATER

MIXING/COMMINUTION
(PHYSICAL TEXTURE MODIFICATION)
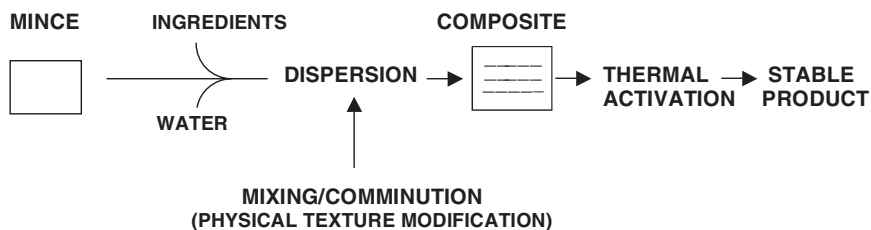
Fig. 13.7 Physical principles of manufacture of formulated fish mince-based products.

All formulated food products are manufactured on the same physical principles, which explain how physical mixing and ingredient variation affect texture and storability (Fig. 13.7). In optimizing a given formulation, one must be concerned about ingredient functions, ingredient-ingredient interaction, thermal activation of ingredients, and fl vour compatibility and interaction of ingredients with fis mince.

13.4.1   Ingredients and processing methods on texture

The functions of ingredients in fis mince products fall into fi e formulation objectives, namely, texture modificatio and enhancement, mouthfeel and moistness improvement, freeze-thaw stabilization, colour whitening, and fl vour modificatio and enhancement. Texture modificatio and enhancement may be achieved by three groups of proteins:

Group 1: egg white, soy protein isolate, and wheat gluten – to improve binding and texture firming
Group 2: textured soy protein (TSP) – to improve bite and mouthfeel;
Group 3: milk protein and soy protein concentrates – to improve water binding with slight texture firming

Texture modificatio can be achieved not only by ingredients, but also by physical means based on mixing and comminution variations. Mixing is commonly done in a bowl mixer or ribbon blender, while comminution is done by chopping in a blade-cutter such as a silent cutter. Mixing without cutting tends to preserve the muscle texture as in products such as nugget, patty, and sausage, while chopping results in emulsion or gel-like texture as in the frankfurter and bologna type products. Texture modifying effects of various proteins in red hake fis mince (unwashed), after bowl mixed and silent-cutter chopped, have been compared under three different cooking conditions using compressive and shear forces for the degree of binding (cohesiveness) and hardness (or chewiness), respectively [30].

The type of protein, mixing method, and cooking conditions greatly affect textural properties [6]. In general, soy protein isolate and egg white yield greater texture strength than whey protein concentrate and milk protein isolate. Among heating schedules, 60 to 80°C results in the lowest texture strength, especially the compressive force, presumably due to the texture softening effect of a slow heating rate combined with the proteolytic activity present. Compressive force (indicative of cohesiveness) is more responsive to mixing effects than shear force (hardness or chewiness). Silent-cutter chopping results in greater compressive force, while bowl mixing yields greater shear force under most cooking schedules. The

pattern of force-deformation response to the processing factors is clearly different between compression and shear tests [30].

The sequence of ingredient incorporation also affects textural properties, depending upon the type of ingredients, especially those having a strong water binding ability (e.g. nonfis proteins), which tend to compete for water molecules with fis mince protein and those which promote hydration (solubilization) of fis mince protein (e.g. salt). For instance, whether adding salt early or later will affect the resulting texture, namely, gel-like texture developed upon adding salt in the early stage of mixing due to extensive protein solubilization and meaty texture, upon adding salt last due to lack of protein solubilization.

As for those ingredients (e.g. potato powder) that undergo thermal activation, proper cooking, particularly in precooking (par-frying for nugget products), is critical for bringing about a desired texture and mouthfeel. Lack of thermal activation, caused by inadequate cooking, results in undesirable mouthfeel, such as coarse and dry sensations. The possible reason for this might be starch retrogradation resulting from inadequate cooking and subsequent freezing.

In addition to textural properties, mouthfeel and moistness are other important sensory attributes that determine the acceptability of the formulated composite product. Most composite products lack juiciness due to fin dispersion of moisture in the matrix, whereas the moisture in the undisturbed muscle tissue exists in the form of a large pocket. Increasing the moistness or juiciness of composite foods could be a technical challenge without addition of fat or oil. As indicated above, formation of large water pockets might be a key solution to this problem. To increase the moistness based on this concept, the product may be formulated such that large ice crystals are formed during frozen storage with addition of water binding but poor freeze-thaw stable ingredients (starch or protein) that hold water initially and allow ice crystal formation later. Formation of large water pockets could also be achieved physically by delaying water addition, such as adding water towards the fina stage of mixing. We found that addition of cheese not only complements fis fl vour, but also improves moistness. In our recent study to improve the moistness in mackerel nugget products [31], we found that milk was more effective than water in rendering moistness and tender texture in mackerel nuggets. Diced vegetables such as onion and mushroom were effective in forming and making the cooked product moist, with less liquid added by holding moisture release during forming and allowing liquid cells to form after cooking, where excess water will make the batter too soft for the forming machine to work with. Milk not only provides moisture, but also serves as a fis y fl vour neutralizer.

13.4.2 Freeze-thaw stability of uncooked mince-based products

Some considerations should be given to freeze-thaw stability of uncooked mince-based products during frozen storage and temperature abuse during distribution, especially for mince prepared from lean fis species, which are known to have poor freeze-thaw storability as evidenced by a high freeze-thaw drip loss and texture hardening. Three groups of ingredients may be considered to improve freeze-thaw stability, namely, nonfis proteins (egg white, soy protein, and milk protein) in combination with sorbitol, STPP, and Ca^{++} chelating alginate [22], modifie starch (primarily hydroxypropylated), and hydrocolloid gums (synergized iota-carrageenan) [32]. Protein with sorbitol and STPP is for those to be uncooked-frozen stored, while starch and gum are for those to be cooked-frozen stored. The mechanism for improvement of freeze-thaw stability by nonfis proteins was proposed earlier by Yoon *et al*. [17], that nonfis proteins stabilize the myofibrilla organization by reducing freeze-induced

shrinkage of myfibrils The freeze-thaw stabilizing mechanisms for modifie starch and gum was explained by Lee *et al*. [33] that use of hydrodynamically active, but unretrogradable starch and gum reduces freeze-thaw drip and subsequent texture changes due to freeze syneresis [34]. The preventative role of alginate in texture hardening is believed to be related to its ability to chelate calcium ions responsible for cross-linking and interfere with muscle fibr interaction.

13.4.3   Colour management

Colour of the meat, especially after being cooked, is important to consumer acceptability. The lighter the colour, the greater acceptability the product will receive. The dark fles fis does present a colour problem. Logically, a certain type of ingredient such as egg white will significantl lighten the colour of the meat. Egg white is an ideal ingredient not only for colour whitening but also for improvement of texture and freeze-thaw stability. Caution must be exercised that colour whitening ingredients be added after removal of dark fles lines for such fis as mackerel. Inclusion of the dark fles will cause problems that are associated not only with darkness of the meat, but also with development of fis y and rancid fl vours.

13.4.4   Flavour enhancement

Flavour is another important sensory attribute besides texture and colour. The control of fl vour profil by use of appropriate combination of fl vour-imparting ingredients is a key to successful products. There are three basic approaches to be made for fl vour optimization in formulation. They are fl vour modification fl vour enhancement, and fl vour masking or neutralization. Flavour optimization requires control of intensity at the right fl vour profile good compatibility between added and indigenous fl vours, and knowledge of fl vour contributions from added ingredients and interaction between fis fl vour and ingredients. Commercially, fl vour incorporation is done by direct addition into the mix (internal use) or external use of fl voured batter, breading, or oil. For example, cheese has good compatibility with fis fl vour. Some fis species such as tuna, salmon, and mackerel have a strong but desirable fl vour compared to white, lean species such as cod and pollock. Washing in an attempt to remove fis fl vour was found to be a wrong approach, since the product becomes quite bland after washing. Therefore, fis mince-based products should be prepared with unwashed fis mince, which carries the fl vour unique to that fish Most fis possess their own characteristic fl vour, which is generally pleasant as long as they are handled and prepared properly.

13.4.5   Application of surimi-fish mince blend in fish cake and kamaboko products

In an effort to extend the utilization of fis mince, the application of surimi-fis mince blend in fis cake and kamaboko type product development is proposed. Unlike surimi, this type of product provides characteristic fis fl vours desirable for the finishe products with more nutrients retained, offers significan cost reduction compared to the formula requiring surimi only, and allows textural fl xibility required by various product lines.

   In a previous study on surimi-fis mince blend [15], red hake (lean white) and Atlantic herring mince were cryostabilized with 4% sorbitol + 2% SPC + 0.4% alginate + 0.3% STPP and 4% MPC, respectively. MPC was added primarily to control lipid oxidation, thus

preventing development of fis y and rancid odour. The mince was blended with Alaska pollock surimi at levels of 0, 20, 40, and 60%. The resulting blends were chopped after addition of 2% salt, 6% modifie tapioca starch (Nustar, Tate & Lyle, formerly A.E. Staley), and water to bring the fina moisture to 78%, and cooked (set at 38°C for 30 min and 90°C for 15 min). There were insignifican changes in whiteness, except for 60% inclusion of fis mince, while marked increases in yellowness were observed in all blends with more pronounced increases in herring mince blends. As for texture, there were steady reductions in both shear and penetration forces with increases in fis mince inclusion, as well as with extended frozen storage. The sensory analysis confirme these changes when rubberiness, firmness and overall liking were evaluated. Based on the combined results, 20% inclusion of fis mince from both red hake and herring resulted in acceptable gel products in terms of colour, expressible moisture, and texture. As for fl vour characteristics, the umami note was greatly improved by adding fis mince, which provides its natural fis fl vours desirable for the oriental fis cake and kamaboko type products.

13.5   Conclusions

The recent marked decline of ground fis stocks calls for effective utilization of the existing fis resources and processing by-products, which becomes increasingly vital to the survival of the present seafood industry. Concerted efforts must be made to develop innovative processes and formulation techniques for conversion and cryostabilization of under- and less-utilized fis species into truly marketable quality products. Successful development of processes and products from fis mince requires a good understanding of physical and fl vour characteristics of meat, ingredient functions, and the basics of food product formulation.

13.6   Acknowledgements

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14 New trends in species identification of fishery products

Hartmut Rehbein

14.1 Introduction

In the last decades, the tools for species identificatio of fisher products have been improved to such an extent that nowadays one can expect to be able to identify each type of fisher product, provided that a DNA sequence is available in the GenBank or related data bases [1]. Meanwhile protein electrophoresis has largely been replaced by polymerase chain reaction (PCR)-based methods of DNA analysis (Chapter 24).

In this chapter, the possibilities offered for authentication of fisher products by two new developments, microarray technology and mRNA analysis, are discussed. Another section deals with methods to identify fis and other seafood. The necessity to identify fis in compound products is given by the high allergenic potential of seafood. Recent legislation calls for rapid and sensitive methods to detect fis and shellfis [2].

Commercially important fis and shellfis comprise several thousand species. Data bases containing DNA sequences and restriction fragment length polymorphism (RFLP) or single strand conformation polymorphism (SSCP) patterns, as well as easily obtainable reference material, are needed for rapid and reliable identificatio of products made of unknown species. Due to the increase in global trade and illegal, unreported and unregulated fisherie (IUU) [3] products appear on the market at the present time, which cannot be identifie as the sequences obtained do not correspond to any sequence in the GenBank.

14.2 Background information

One of the main factors determining the price of a fisher product is the animal species being processed. For example, different species of tunas (yellow fi tuna (*Thunnus albacares*), blue fi tuna (*Thunnus thynnus*), and albacore (*Thunnus alalunga*)) are achieving prices in retail shops or sushi restaurants, which may vary considerably. Due to over fishing a number of commercially valuable species can no longer meet the demand of the market. After the morphological characteristics of fis are lost during processing, identificatio becomes very difficul for experts and impossible for the consumer. Against this background it is not surprising that mislabelling of fisher products is widespread at the present time, as many

Table 14.1   Recent publications about mislabelling of fishery products

Summary	Number of cases of mislabelling	Reference
Comprehensive review about renaming and mislabelling of seafood.	It is estimated that $>\frac{1}{3}$ of fish sold in the US are mislabelled.	[3]
Fish meals from restaurants, school, and university cantines were analysed by PCR for grouper (*Epinephelus marginatus*) as indicated on the menu.	28 out of 37 samples (76%) were incorrectly labelled.	[43]
Mislabelling of oil, fish, and escolar as codfish caused 600 cases of keriorrhea in Hong Kong in 2006.	13 out of 30 samples (43%) were mislabelled.	[6]
Fish sold as Pacific red snapper was identified by PCR and sequencing as rockfish (7 species) and tilapia.	Of the 77 samples analysed only one was true red snapper (*Lutjanus campechanus*).	[44]
Surimi-based products labelled as Alaska pollock (*T. chalcogramma*) were analysed by cytochrome b PCR and sequencing.	Of 19 products tested 16 were mislabelled (84%).	[45]
DNA barcoding was used to analyse 91 samples of North American seafood	23 of 91 samples (25%) were suspected to be mislabelled.	[46]

reports of food control authorities are demonstrating. A number of recently published studies dealing with the subject are compiled in Table 14.1. Illegal IUU fishing which has become a billion dollar business, has to be regarded as another important source of mislabelled fis [4]. Development of rapid, reliable, and low-cost methods for fis species identificatio is necessary in order to:

- protect the consumer against fraud;
- protect the consumer against health risks; e.g. such risks may be caused by labelling escolar fis such as sea bass [5] or cod [6];
- safeguard fair trade and to combat IUU [4];
- support correct tariffin of fisher products by customs authorities [7];
- protect endangered species against depletion of stocks or extinction of populations;
- improve catch statistics [8].

14.3   Microarrays

DNA microarrays consist of hundreds or thousands of spots of cDNA (transcribed RNA) or printed oligonucleotides bound to a matrix (e.g. coated glass slide) to be hybridised with segments of DNA obtained by PCR. In fis and shellfis research, DNA microarrays have become an important tool for studying gene expression under different physiological and environmental conditions [9]. Differentiation of fis populations by single nucleotide polymorphism (SNP) arrays may become an attractive alternative to microsatellite analysis in the near future [10].

The construction of DNA microarrays (FishChip) to be used for authentication of fish ery products has been delayed by lack of sequences of commercially relevant fis species. However, in the near future the situation will change, as the Fish Barcoding of Life Initiative

(FishBol) is making good progress to deliver sequences of part of the cytochrome ox-idase I – gene for all of the 29,000 fis species existing in marine and fresh water (http://www.fishbol.o g). Up to now (December 2008), 20% of fis species have been se-quenced. At the same time, the number of sequence entries in the GenBank for other genes of fis (cytochrome b, 16S rRNA, nuclear genes) is continuously growing.

In the last few years, several papers have been published describing the development and firs applications of different DNA microarrays for identificatio of fish birds, and mammals. Chisholm *et al*. [11] reported their experiences with the bioMerieux FoodExpert-ID$^®$ system used to analyse animal feed and meat mixtures. The chip contains probes (cytochrome *b*) of 12 mammals, 5 birds, and 16 fis species. At first a cytochrome *b* sequence of the sample DNA has to be amplifie by PCR. Then, transcription of the amplicon and simultaneously labelling with a fluorescen dye, the RNA produced is enzymatically cleaved, followed by hybridisation of the fragments to the microarray. The authors concluded that for mixed products (meat, canned meat, and feed) of warm-blooded animals, the bioMerieux FoodExpert-ID$^®$ system was suitable as a screening method to identify various species present in a sample if their level did not fall below a threshold, depending on the type of product and the species to be identified However, results for fis have not yet been found in the literature.

In another study, cytochrome *b* gene-derived probes (oligonucleotides) had been used to develop a microarray-based method for identificatio of 77 species of fis and other vertebrate species [12]. The length of the oligonucleotides bound to an aldehyde containing microarray slide varied between 17 and 24 bases. The analytical procedure to identify the species in products containing a single or a number of species comprised the following steps.

- Amplificatio of a sequence of the cytochrome *b* gene (124, 278, or 373 bp) using universal primers. One of the primers of each pair had been phosphorylated at the 5′-end, the other one was labelled by Cy3.
- After PCR, the phosphorylated DNA strand of the amplicon was degraded by treatment with λ exonuclease, the remaining Cy3-labelled strand was hybridised to the microarray.
- By means of a scanner the fluorescenc intensity of the spots was measured. If fluorescenc intensity was 20% above the mean intensity of the blanks, the result was considered to be positive.

The whole procedure took two days. This technique allowed reliable identificatio of 71 out of 77 species and was found to be especially useful for testing of mixtures. Analysis of mixtures by sequencing, RFLP- or SSCP-analysis of amplicons needs a cloning step in most cases. Development of the method was hindered by a few wrong sequences deposited in the GenBank.

Kochzius *et al*. [13] selected a 600 bp fragment of the mitochondrial 16S rRNA gene for their microarray experiments. The DNA chip was constructed by spotting oligonucleotides of 23–27 bases onto aminosilane-coated glass slides. PCR products were labelled with 5′-Cy5-modifie primers. By following a similar procedure as described above, they succeeded to differentiate 11 fis species.

Further progress in identifying fis by DNA microarrays may depend on the cost for using DNA chips (equipment and consumables) compared to the cost for sequencing. Due to progress in sequencing technologies, DNA sequences have become very cheap [10]. As mentioned above, DNA sequences of all fis species will become accessible in the next few years. They can be used to identify any unknown fis species by performing PCR with universal primers and sequencing of the amplicon.

14.4   Messenger RNA analysis

At firs sight, usage of mRNA for species identificatio seems to be limited by tissue specifi expression and low stability of mRNA. Concerning the firs point, differences of expression between tissues may be large, with the exception of so-called stably expressed "housekeeping" genes used as controls in mRNA expression studies [14]. However, ubiquitous tissue expression of mRNA has been observed not only for housekeeping genes, but also at a low level for a number of inducible genes [15]. Obviously a pool of non-completely degraded mRNA exists in many tissues, resulting from unspecifi or illegitimate transcription [16].

In vertebrate tissues, short sequences of mRNA could be detected by end-point reverse transcription (RT)-PCR or quantifie by real-time RT-PCR. In forensic casework analysis, mRNA profilin has been used to identify the origin of body fluid or tissues (blood, semen, saliva, and epithelial cells) [17,18].

Messenger RNA has also been identifie in different fisher products. By means of RT-PCR, Rehbein and Kreß [19] detected short sequences of parvalbumin and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (length: 120–250 bases) in raw, cooked, smoked, or canned fille of salmonid, and gadoid fis species. Later, these finding were corroborated by real-time RT-PCR, which demonstrated the high resistance of parvalbumin mRNA fragments against heat and acid [20]. Recently, a study was published showing occurrence of gene expression, for example, mRNA *synthesis*, in fis muscle stored in ice for 24 hours [21]. Messenger RNA of troponin I and GAPDH were detectable by real-time RT-PCR for at least 72 hours post-mortem.

Applications of mRNA analysis in fis species identificatio are to be seen in cases of products being rich in RNA, such as food made from fis roe (e.g. caviar and tarama) [22], or in marinated products possessing a low pH value (<4.8), such as Bismarck herring and rollmop (rolled pickled herring). RNA of marinated herring seems to be more stable than DNA (Rehbein, 2008, unpublished results).

Differentiation of populations of fis and shellfis by mRNA profiling of "biofish and conventionally farmed fis by RT-PCR of stress-induced mRNA [23], identificatio of fis from polluted waters [24], and detection of allergenic proteins (see next section) are other potential applications of mRNA analysis (transcriptomics).

14.5   Detection of allergenic fish and shellfish

Fish, shrimps, and mussels have a certain allergic potential as other foods. Once established, seafood allergy is usually a life-long problem for persons affected. According to the directives and regulations being in force in many countries, fish crustaceans, and molluscs, and products thereof, must be indicated in the list of ingredients to give allergic consumers the chance to avoid seafood [25].

Immunological as well as PCR-based methods have recently been developed to detect fish crustaceans, and molluscs in composite products such as pastries, pates, cakes, or soups. The most important allergenic protein in fis muscle is the water-soluble, small molecular mass (∼12 kD), and calcium-binding parvalbumin [26], whereas the myofibrilla protein tropomyosin is responsible for most of the allergic reactions against shellfis [27].

Recently, a quantitative sandwich ELISA for the determination of fis in foods has been developed and validated. The test system consists of a polyclonal rabbit anti-cod parvalbumin
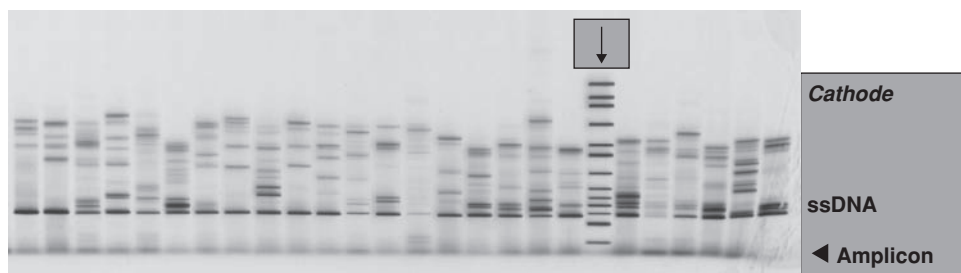
Fig. 14.1 Detection of parvalbumin mRNA and differentiation of 25 fish species by SSCP analysis using native polyacrylamide gel electrophoresis with silver staining. ↓: DNA size standard (base pair ladder).

antibody for capture and a biotinylated conjugate of the same antibody for detection [28]. The test is sensitive (limit of detection: ∼5 g fish/k of food) and highly specifi for fish However, some species (sturgeons, Northern pike, and anchovy) do not react. A number of tropomyosin-based ELISA for detection of shellfis is now commercially available [29].

PCR-based assays have become an interesting alternative for detection of the presence of a certain organism in food [2]. In the case of fish any fish-specif PCR must work efficientl with DNA of several thousand fis species used for human consumption, but should not react with DNA of other animals. Nevertheless, PCR-based test systems for detecting "fish" "crustaceans", and "molluscs" as classes of organisms have been developed [30], already commercialised [31,32], and proposed as officia methods [29]. However, it should be kept in mind that the results of PCR must be interpreted with caution, as PCR detects the presence of DNA, not of the allergenic protein itself.

As described above, measurement of the amount of mRNA (gene expression) has completed determination of enzyme activity or protein concentration in fis physiological studies [23,33]. The technique of RT-PCR is sensitive, relatively easy to standardise, and suited for quantitative assays by using real-time PCR.

The discovery of an unexpected stability of short sequences of mRNA in fis muscle offered the possibility to develop new methods for allergen analysis by determination of parvalbumin mRNA in fisher products [19]. The stronger relation between protein and the mRNA, compared to the relation between protein and DNA, can be seen as an advantage of RT-PCR against PCR in allergen determination. Figure 14.1 shows the results of a combination of RT-PCR and SSCP analysis to identify parvalbumin mRNA (allergen indicator) and determines the species within the same protocol.

14.6   Determination of origin and stock assignment of fish

Fishery biologists have been analysing the status of fis stocks for decades. However, the industry, non-governmental organisations, and food control authorities have recently become aware of the necessity to include information about the geographical origin of the fis in traceability systems and food labelling regulations to support sustainable fisheries

The methods to be applied for identificatio of the origin of fis should work with fille and other products, as the market for fresh fis is relatively small. The combination of molecular biological techniques and chemical analysis has a great potential to solve the difficul

problems related to differentiation of populations. The following genetic identificatio methods were found to be suited for differentiation of fis populations [4]:

- microsatellite analysis;
- determination of SNP.

Encouraging examples for the suitability of these methods is the results obtained with Pacifi salmon (*Oncorhynchus tshawytscha*) [34] and Atlantic cod (*Gadus morhua*) [35]. In the future, these methods may be supplemented by mRNA analysis to follow the expression of genes regulated by environmental conditions such as temperature or salinity [36,37].

The chemical composition of fis muscle (fillet is mainly affected by the feed of the fish By measuring stable isotope ratios [38] and fatty acid profile [39], fis of different origin could be separated. Recent progress in metabolomics, for example, by nuclear magnetic resonance (NMR), may offer additional possibilities of assignment of fis to populations [40].

14.7 Data bases

Despite the great progress made within the last two decades in PCR-based DNA analysis to identify fis species in all types of products, it is still difficul for food control laboratories to analyse samples of unknown origin. There are several reasons for this unsatisfactory situation.

- The increasing demand for seafood coupled with the shortage of traditionally consumed fish forces trade and industry permanently to introduce new species onto the market. Quite often, only poor information about these new species is available in the importing countries.
- Reference material is difficul to obtain for food control laboratories. A network of laboratories for exchange of samples does not exist. This problem will become greater, if assignment of fis to origin has to be controlled.
- In the case of fish several data bases compiling DNA sequences, DNA profile obtained by secondary techniques of amplicon characterisation (RFLP and SSCP analyses), as well as background information about samples have been established. These data bases, which were outcomes of projects in most cases, still have large gaps. For crustaceans and molluscs, the situation is even worse, as no data bases exist at all.

Besides the GenBank and the related data bases, the largest data base for fis is provided by the Fish Barcoding of Life Initiative (Chapter 24). Smaller data bases for fis are as follows:

- FishTrace (http://www.fishtrace.o g);
- FishGen (http://fishgen.jrc.it/)
- Amplifie restriction fragment length polymorphism (AFLP) data base (nonnis@biol. unipr.it);
- Validation (www.seafoodplus.org; http://www.azti.es/dna_database/);
- FischDB (www.fischd .de);
- Regulatory Fish Encyclopedia (RFE) (http://www.cfsan.fda.gov/~frf/rfe0.html#cname).

14.7.1   FishTrace

FishTrace, initiated by a project funded by the EU and now located at the Joint Research Centre in Ispra in Italy, is a data base for identificatio of more than 200 fis species from European and adjacent waters. The data base contains information about the biology, DNA sequences (cytochrome *b* (mitochondrial gene) and rhodopsin (nuclear gene)) and analytical tools for each species. Users can enter their data for BLAST or phylogenetic analysis (Phylip).

14.7.2   FishGen

FishGen is a small data base, with data for 11 species available. The data have been collected from the literature, and are not very useful for product analysis.

14.7.3   AFLP

AFLP is a PCR-based technique mainly used in population studies. Without prior knowledge of DNA sequences of the species under study, it is possible to obtain hundreds of genetic markers [41]. AFLP analysis gives more reproducible results than the random amplifie polymorphic DNA (RAPD) method, but is limited by the necessity to use non-degraded or only slightly degraded DNA. Nevertheless, recently a database with AFLP patterns of 32 species of fish molluscs, and crustaceans (frozen and fresh products) has been generated [42].

14.7.4   Validation

This is a sub-project within the large European project SEAFOODplus, which was terminated at the end of 2008, with following objectives:

- to validate the traceability systems developed and implemented in different fis production chains across Europe;
- to validate the traceability data coming from the chains testing different tools, such as PCR-based DNA analysis.

The data base is hosted by the Spanish research institute AZTI. It contains sequences for a number of mitochondrial genes of more than 50 fis species.

14.7.5   FischDB

The aim of FischDB is to support food control laboratories by delivering:

1) consensus sequences of the cytochrome b gene of fis species;
2) tables of fragment sizes to be used in RFLP analysis;
3) figure with SSCP patterns; and
4) protein patterns and pI values obtained by isoelectric focusing of sarcoplasmic proteins. The explanations are in German, thus restricting the usage of the data base.

14.7.6 RFE

RFE consists of a collection of protein patterns obtained by isoelectric focusing (IEF) of sarcoplasmic proteins, tables of corresponding pI values, and images of fishe and of the respective fillets At the moment, data of more than 90 fis species important for the North American seafood market are compiled in RFE.

14.8 Conclusions

A large number of genetic and chemical methods for fis species identificatio are now available to authenticate all kinds of fisher products. However, many of them have not been validated by collaborative studies, and do not have the status of officia methods. Against a background of increasing fraud and illegal fisheries certificatio of methods is necessary to establish their acceptability in the industry and trade, as well as in the courts. A network of laboratories and institutions would be very helpful to coordinate the existing data bases and to improve access to reference material.

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15 An emerging powerful technique: NMR applications on quality assessments of fish and related products

Somer Bekiroğlu

15.1 Introduction

Nuclear magnetic resonance (NMR) spectroscopy provides ease, versatility, and power that has lead to advancements within the life sciences. However, concerning the degree of employment of NMR spectroscopy as a technique in applied sciences and particularly in food science, it is imperative to state that the possibilities have not been exploited to their full extent compared to the ones exploited ubiquitously within chemical and structural analyses areas.

NMR spectroscopy in food science is generally used for studies such as the analysis of solid fat content (fat containing products), moisture content (margarine, starches, and rice etc.), component analysis (milk), moisture and fat content (general food products, e.g. milk powders), oil composition (oils), moisture and oil content (emulsions and flour) droplet size distributions (emulsions), moisture and solid distributions (suspensions and gels), freezing process research (solids and liquids), and extent of cooking/hydrolysis (ready-to-eat/cooked food) [1]. Therefore, it is not difficul to foresee that fis and related products are no exceptions for the upcoming extensive use of NMR techniques. Many different fis and fis product characteristics that can be attained by NMR spectroscopy range from fingerprintin analysis of marker compounds for authenticity studies [2] to the non-destructive analysis of whole fis for quality control efforts [3]. In general, NMR spectroscopy is typically non-destructive and sometimes non-invasive technique that gives varied information about molecular structures, as well as temporal and dynamic molecular properties, in terms of spectra of various dimensions. With a coarse simplificatio it can be depicted as measuring the resonance response of nuclei to applied electromagnetic radiation usually with radiowave frequency (RF), provided that the nuclei possess non-zero spin quantum numbers (e.g. ^{1}H, ^{13}C, ^{23}Na, ^{31}P, etc.) and are placed in a constant magnetic fiel (B_0). These signals carry important structural information about the sensed local environment within the molecule studied. The resonances (the state of emission of radiation absorbed as RF) fade out as time goes by, following two major relaxation pathways, spin-lattice relaxation T_1 and spin-spin relaxation T_2. Usually structures of molecules are studied in a solution environment. However, solid-state NMR and magnetic resonance imaging (MRI) methods allow one to study heterogeneous systems of intact samples such as cells [4] or even tissues, and whole

fis samples [5]. Classifie under three subtitles, low-fiel (LF), high-fiel (HF), and MRI, this chapter highlights different examples of possible NMR applications for fis and related products.

15.2   Low-field (time-domain) NMR applications

The resonating frequency of NMR active nuclei within a constant magnetic fiel is dependent on the magnetic fiel strength (e.g. the higher the magnetic fiel strength, the higher the resonant frequency). When implied as LF-NMR spectroscopy, the magnetic fiel strengths attained usually result in between 2 and 30 MHz proton resonant frequencies. Since most of the LF-NMR applications are dependent on relaxation phenomenon and data in a time domain unlike HF experiments, where time-dependent data (so-called free induction decay, FID) are transformed into frequency domain by the famous mathematical operation Fourier transformation (FT), LF-NMR is also named as time-domain (TD) NMR.

Relaxation and diffusion measurements form the core of LF-NMR experiments. This intrinsically eliminates the requirements to have highly homogenous and strong magnetic field opposing the HF-NMR applications. Therefore, permanent magnet technology without any cryogenic parts would suffice reducing the overall costs of the systems drastically. Recent developments in permanent magnet engineering have made it possible that even unilateral, hand-held mobile sensors can be purchased and easily adapted to different LF-NMR applications [6].

15.2.1   Water, lipids, and others

The idea of LF-NMR applications was firs developed at the beginning of 1970s by a group of scientists from Unilever Research (The Netherlands) and Bruker Physik AG [7,8]. Their aim was to build a small table-top time-domain (TD)-NMR analyzer for the solid-to-liquid ratio analysis on fat compositions. Today, similar instruments are widely adapted and used for the analysis of relaxation and diffusion properties.

LF-NMR techniques can be employed for quick analyses of fat, water, or/and protein by relying on the fact that different compound types (fat, water, and protein) have different relaxation characteristics. This is even true for proton signals detected as the magnetization in different forms (e.g. bound and free water). Once the necessary calibration curves are established for different compound types in various matrices (including fis and related products), to determine their contents becomes a straightforward procedure. The use of this valuable technique can easily be extended to study product and quality related changes faced during fis processing and subsequent storage such as marinating, salting, heating, chilling, freezing, pressure treatment, and modifie atmosphere packaging (MAP) etc. [9–16].

Considering the conventional chemical-physical analysis techniques, the NMR technique proves to have the potential of replacing the classical methods as rapid and low-cost alternatives. Often no or very little sample preparation is needed prior to the analysis. This ultimately results in the fact that the use of chemicals potentially harmful to health and environment chemicals would be avoided. Furthermore, these bench-top instruments are equipped with fully self-adjusting systems without any need for manual tuning of measurement parameters. This, in return, provides ease of use and, thus, highly skilled laboratory personnel would not be required. Usually the LF-NMR spectrometers have internal probes that can

accommodate NMR tubes of 5 to 50 mm in diameter. This brings about the relative difficult of working with pre-treated (cutting, mincing, homogenizing, etc.) samples, which should fi the restricted bore size instruments. Initially pioneered for the applications of material testing in 1995 by the group of Professor B. Blümich [17], recent developments in probe technology and elaborated pulse sequences have also allowed the development of one-sided or unilateral probes that proved to be useful, even for fat content determinations of intact fis samples [18]. Such hand-held NMR sensors allow measurement of NMR relaxation and diffusion parameters in surface-near volume elements of arbitrarily large objects.

Analysis of fat, water, protein, or any other type of compound, whose protons (^{1}H) have characteristically different NMR relaxation properties, can be performed using LF-NMR techniques. Although being dependent on the method and type of LF instrument, the analyses firs require acquisition of relaxation profile of the sample to distinguish the signals of the compounds in question. The TD-NMR techniques in food sciences and industry are typically used for the products containing either an ample amount of water or fat with the purpose of determining the concentration or the amount of a specifi component, respectively. The restriction to either low water or low fat products is due to the fact that in TD-NMR almost no spectral resolution is available and that the transverse and longitudinal NMR relaxation properties of fat and water molecules are not substantially different [8]. However, there are attempts to overcome this difficult   as various multivariate analysis methods and the diffusion weights are applied [19] or two-dimensional methods are employed that correlate distributions of relaxation times and diffusion coefficient [20,21]. Following the relaxation time profiles calibration curves against known concentration samples are prepared. This way any new measurement would allow one to determine the content of the compound in question. In Fig. 15.1, an example of such a spectrum (a) and respective calibration curve (b) for the fat content analysis of Atlantic salmon (*Salmo salar*) can be seen. For this work, Veliyulin *et al.* [18] made use of a one-sided probe on intact fis samples. When the results from the NMR study were plotted against the data from the chemical analysis of the same samples (NMR results on live fis vs. chemical analysis results on the same samples after slaughtering) a good correlation coefficien (r^2) of 0.92 was easily achieved. They found
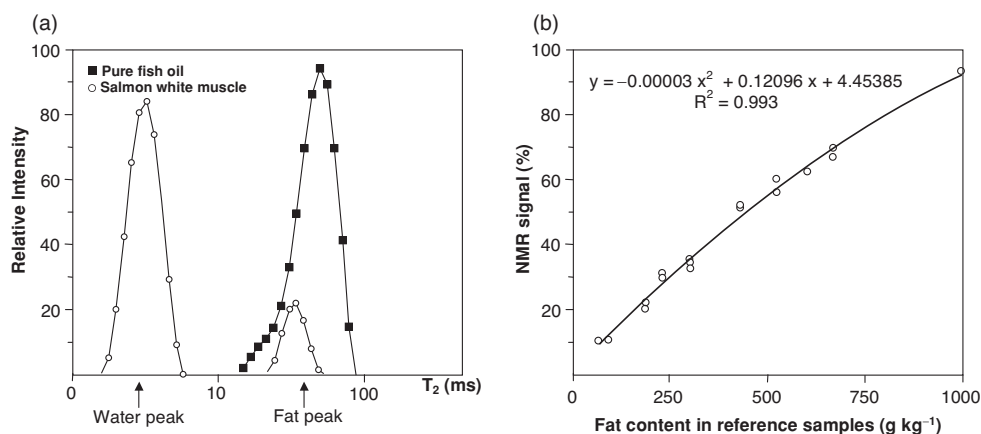
Fig. 15.1   (a) Relaxation spectrum of salmon sample and pure fish oil, and (b) the calibration curve with respect to the reference samples of known fat concentration. Adapted from Veliyulin *et al.* [18], with permission of Blackwell Publishing Ltd.

that the average standard deviation of the differences between the two methods was 1 g/kg with the mean error in the 95% confidenc interval of 19 g/kg. For salmon with similar body weights (∼4 kg), the fat content values (90–180 g/kg) reported in this study can be considered as typical for this size of salmon. As another example, applying multivariate analysis on relaxation data, Jepsen *et al.* [13] similarly determined fat and water content as well as water holding capacity in fis muscle.

LF-NMR can also be used to determine the effects of storage conditions such as frozen and chilled. Lambelet *et al.* [12] studied the effects of frozen storage of cod by exponential curve fittin on the NMR relaxation data. Jensen *et al.* [22] studied the effects of both chilling and freezing storage by employing a multivariate three-way modelling of relaxation profiles Examples of analyzing the distribution of water in cod [11] and in herring [23], as well as texture changes in frozen cod [24], can also be found in the literature.

15.2.2   On-line and off-line applications: quality control

High-quality products are among the most important priorities for fisherie and aquaculture. Therefore, the need for quick and non-destructive methods for quality control (QC) is growing steadily in line with the demand from the industry. NMR spectroscopy has massive potential to satisfy the quality control needs required in fisherie and related industry plants. When it comes to cover the range of various analyses, NMR spectroscopy is certainly superior to its alternatives, such as traditional chemical methods and near infrared (NIR) techniques [3]. However, current NMR applications are mostly done as at-line analyses. The main reasons for this preference are that NMR methods at present would not match the speed requirements of some production lines and that at-line analyses cover most industrial needs. Intriguingly more and more sophisticated instruments are being released. Almost all of the instruments are made to satisfy certain specifi needs of the industry. For example, apart from the already mentioned moisture, lipid, and protein cases, major LF-NMR instrument producers have ready-to-use solutions for determination of polar parts in deep-frying oils, water distribution in dispersions and gels, droplet size distribution in water in oil emulsions, and investigation of freezing processes, etc.

As an illustrative example, LF-NMR spectroscopy QC applications on fis feed production can be given. It is worth mentioning that controlling the composition and the quality of fis feed is absolutely essential and directly influence the economies of both fis and fis feed producers. At-line analyses performed on the product line cover almost the whole range of parameters to be checked. The raw ingredients of the product constitute mainly proteins, carbohydrates, moisture, and fat, all of which are possible target compounds for LF-NMR applications [25]. Thus, LF-NMR offers a wide range of relatively quick, non-destructive, and accurate methods. Such checkpoints can be inserted anywhere on the production line. An illustration showing the possible stages of these analyses is given in Fig. 15.2.

15.3   High-field NMR applications

The underlying principles of HF-NMR spectroscopy have already been explained in the previous section. The main difference compared to LF-NMR (usually proton frequencies between 2 and 30 MHz) is the strength of the constant magnetic fiel reaching up to 22.3 Tesla with the contemporary magnets of 950 MHz proton resonance.
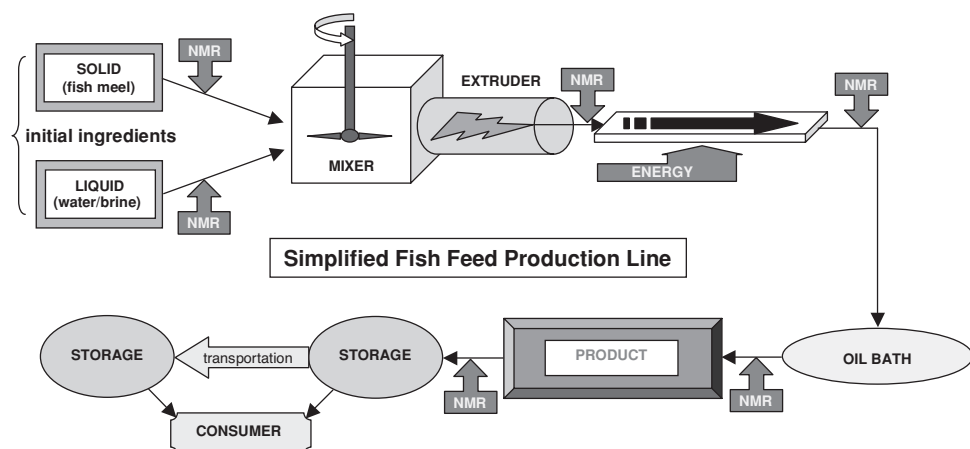
Fig. 15.2 Application of low field NMR in quality control during fish feed production. Adapted from SINTEF [25], with permission.

Studying fis and related products by HF-NMR spectroscopy follows the same pattern of other HF-NMR investigations used for structure, dynamics, and quantitation efforts in chemistry, biochemistry, and similar disciplines. The reason for this is that, once the subject material (sample) is separated from the original matrix, the NMR routines of various techniques are all the same. The same applies to the so-called high resolution magic angle spinning, HR-MAS technique (an HF-NMR method for semi-solid matrices), by which target molecules in intact samples of cells or micro-organisms can be analyzed [26].

15.3.1 Quantitative NMR applications and chemical compositions

By definition NMR spectroscopy can be considered as a primary ratio method of measurement since the peak areas are proportional to the number of corresponding nuclei giving rise to the signals. Provided that the suggested protocol is applied for the determination of molar ratios, the uncertainty value calculated for a quantificatio measurement by NMR spectroscopy has been found to be 1.5% at a 95% confidenc level with k = 2 [27]. This value is similar to those of the alternative analysis methods. With today's modern high-fiel instruments, it is possible to study compounds at concentrations as low as a few microlitres solutions in the order of approximately 100 μM. The exact detection limit cannot be given because it is dependent on the NMR characteristics of the studied compound and can easily be improved by the NMR sampling time (i.e. longer experiments and more number of scans).

Together with quantitative (q) NMR applications, further assessments of metabolites (biomolecules, as well as xenobiotic molecules of various sizes) are straightforward routines, supplying a wealth of various sorts of properties of the studied compounds with a single shot analysis in the form of structural, physical, and quantitation information. Of course, the versatility of NMR spectroscopy plays an important role in these kinds of augmented studies. To exemplify, qNMR studies of glycine-betaine in mussels [28] and betaine, as well as other bioactive compounds in cod [29] can be given. There are also many other

metabolites in fis muscle that might be of interest concerning the quality and nutritional evaluation of fis and related products. These metabolites such as lipids (triacylglycerols (TAG), phospholipids, cholesterol, and free fatty acids (FFA)), phosphocreatine, various nucleotides (e.g. adenosine-5′-tri- (ATP), -di- (ADP) and -mono phosphates (AMP) etc.), taurine, anserine, dimthylamine, trimethylamine oxide can be studied to determine their contents in relation to their correlations with growing, processing, and storage conditions. Identification of such low-molecular-weight metabolites in extracts of salmon [30] and cod [31] have been performed by ^{1}H HF-NMR spectroscopy. The same approach has also been used to follow the changes in fis products at some stages in freezing [29,32], as well as in thawing and cooking [29]. The biochemical changes (hydrolysis and esterification in lipids of cod (*Gadus morhua*) throughout a storage period of 7 days at 4°C have been studied by ^{13}C HF-NMR [33]. The analysis of fis oil to determine the content of docosahexaenoic acid (DHA) and omega-3 (n-3 or ω-3) fatty acids have been performed using the ^{1}H HF-NMR method [34,35]. Another major application area for HF-NMR would be the employment of phosphorus (^{31}P). Whenever ^{31}P is present within a molecule, it can be considered as a well-behaving subject to NMR analysis. Therefore, phospholipids, among other ^{31}P containing biomolecules, have also been studied extensively, and the relevant literature has been reviewed by Schiller and Arnold [36].

15.3.2 Fingerprinting

Many instrumental methods in chemistry make use of fingerprintin as a widely employed technique. This method is also frequently used in food NMR context in which unique NMR spectra of individual samples are employed to analyze compositional, quantitative, process-related, and origin-related changes. Mostly fingerprintin methods are directly associated with metabolic profilin and, therefore, are thought of as HF-NMR applications. However, concerning the quality control issues, this method can be employed in LF-NMR and MRI studies as well.

The simplest example would be on-line fingerprin analysis as a part of a QC attempt on a production line, in order to check whether the analyte matches the unique fingerprin pattern of the product within normally accepted limits. The calibrations of such limits can easily be done using other chemical and physical methods, as well as other NMR techniques. Similar to the applications in pharmaceutical industry, we can now imagine a fis oil producing plant or a fis oil refiner where, at certain points in the production line (Fig. 15.2), samples can be taken and used to obtain simple one-dimensional (1D) HF-NMR proton spectra. No matter how complicated the spectra are the method can easily detect the changes compared to the ones accepted as normal. Any unexpected deviations from the normal spectra (the resulting spectra of the accepted calibration limits) would give subtle reporting about possible quality problems, which can easily be subjected to deeper analysis.

Fingerprinting followed by multivariate analysis is frequently used to overcome the diffi culties in handling samples giving complicated spectra. These are usually metabolic fluid or extract from different living organisms. By far the most extensively used multivariate method in NMR data mining is the principal component analysis (PCA), where a smaller set of synthetic variables are sought. This way, the interdependency of the variables is explained. Its ease of use and simple visualization possibilities are the key elements in its broad use. Other multivariate techniques, such as linear discrimination analysis (LDA) and probabilistic neural networks (PNN), are also attained.

15.3.3 The future: fish metabon(l)omics

Pursuing scientifi information about the metabolic status of organisms, it is possible, with the help of NMR spectroscopy, to reveal the consequences of diseases, toxic insults, genetic manipulations, and environmental changes. Determining the metabolic status of an organism, that is, understanding its metabolome (complete set of small molecule metabolites) is exactly what is achieved by metabolomics. Although both terms are used synonymously, metabonomics, on the other hand, is a concept that is supposedly related to the quantitative profilin (not comprehensive though) of multi-parametric and simultaneous metabolic changes caused by biological perturbations.

As is true for other foodstuffs of living origin, metabon(l)omics studies should be considered as one of the cornerstones in opening the door of a new era, in which many important quality and nutritional parameters of fis and related products can be controlled proactively. The knowledge acquired by metabon(l)omics studies on basic physiology and development of fish effects caused by diseases and environmental conditions (e.g. water pollution and exposure to toxins, etc.) would furnish invaluable tools for both the production and consumption related problems. Once the key factors in the evolution of a problem are known, it is, of course, easier to fin a solution. For instance, Viant *et al.* [37], in their study of juvenile steelhead trout (*Oncorhynchus mykiss*) by ^{1}H NMR, revealed that thermal stress causes a decreased metabolic condition (lower phosphocreatine, ATP, and glycogen levels). In the same line as Viant *et al.* [37], Karakach *et al.* [38] studied the metabolic response of Atlantic salmon to long-term handling stress and could see that even one week of stressful handling was reflecte in metabolic reporters. Southam *et al.* [39] could detect the metabolic differences between healthy and hepatic tumour tissues in flatfis proving elevated anaerobic metabolism and reduced choline metabolism in tumour tissues.

Metabolic changes over toxic exposures can also be studied by NMR spectroscopy. Using simple 1D ^{1}H NMR spectroscopy in conjunction with PCA, Ekman *et al.* [40] could identify taurine, lactate, acetate, and formate in urine samples as metabolic markers of vinclozolin (a common fungicide) exposure in the male fathead minnow (*Pimephales promelas*). Metabolic effects of dinoseb, diazinon, and esfenvalerate in eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) have been determined by ^{1}H NMR metabolomics [41]. The metabolic effects of dinoseb in Japanese medaka (*Oryzias latipes*) embryos *in vivo* were determined by Viant *et al.* [42]. Samuelsson *et al.* [43] could identify vitellogenin, alanine, phospholipids, and cholesterol as the main affected metabolites in blood plasma and plasma lipid extracts from rainbow trout exposed to the synthetic contraceptive estrogen ethinylestradiol (EE2) with plasma from control fish As the concluding remark on metabon(l)omics studies of fish it is certainly worth mentioning that Samuelsson and Larsson [44] have recently reviewed metabolomics studies in fis research, providing a comprehensive summary of the field

15.3.4 NMR and authenticity

Authenticity of food and food ingredients has always been at the centre of scrutiny of producers and processors, as well as the consumers. NMR spectroscopy is deeply rooted in the history of authenticity studies in food sciences. The basic principle is always the same and boils down to the point that we need to fin easily and reliably detectable/measurable parameters that can differentiate the foodstuffs and help us to trace/identify their origin. When NMR spectroscopy is considered in the context of authenticity, many parameters are possible to adapt in conjunction with the use of advanced data analysis tools.

The most important footstep in food authentication by NMR spectroscopy goes all the way back to the pioneering work of Professors Gerard and Maryvonne Martin in the early 1980s. They adapted the idea based on the specifi distribution patterns of stable isotopes and named the method as site-specifi natural isotope fractionation by NMR spectroscopy, SNIF-NMR®. The method, which was in fact initially developed for the authentication of wine, is now used for many different foodstuffs including fis and related products. Using SNIF-NMR® and lipid profiling it is now possible to obtain information about the geographical and wild/farmed origin of the fish as well as about the species and breeding stock [45]. However, the present NMR methods for fis authentication should still be improved, since it is not yet an internationally accepted, validated, and consistent method that emerged from the attempts made so far [46]. These attempts, among many others, include the study of ^{1}H NMR data evaluated by using advanced data mining methods that allowed a successful identificatio of wild and farmed salmon [2]. For fis oil and related health products, Aursand *et al.* [47] showed that ^{13}C NMR data, as examined by multivariate analysis techniques, could provide information about the nature, composition, refinement and adulteration of the products. There are also other studies, where fatty acid positional distributions in TAG [48] and in phospholipids [49] have been shown to provide possible authentication information.

15.4   Projections on MRI applications

With the numerous and invaluable opportunities instigated by MRI in medical sciences, today almost everyone is familiar with the MR images of different body parts. MRI, especially due to its non-destructive character, is a unique technique that can produce information-rich images characterized by relaxation properties, chemical structure, and mobility, and cross-sectional images of intact parts of living organisms. In addition to structural information, this powerful tool can be used to obtain temporal and dynamic changes in different subjects.

Similar to the applications in medicine, anatomical studies of fis and other aquatic organisms are easy to perform. Initially, Blackband and Stoskopf [50] showed an example of such an MRI study, followed by the ^{31}P and ^{1}H MRI investigations by Bock *et al.* [51]. Later Veliyulin *et al.* used MRI to detect backbone deformations in farmed salmon [5] and to prove that the stomach was not the source of enzyme release causing belly bursting in herring [52].

"Diffusion weighing" is one of the MRI methods that are used to obtain high contract images, depending on the mobility of the molecules within the sample tissue. Using strong longitudinal relaxation time (T_1) weighing, images differentiating adipose and muscle tissues could be obtained [53]. However, better suppression of the signals originating from either water or fat molecules is required for quantificatio purposes. This could be achieved by using selective excitation pulses [5,54]. This way, accurate quantificatio of water and fat content becomes feasible. The resulting images of this approach can be seen in Fig. 15.3, showing pieces of salmon fillet

Acquiring similar MR images of NMR active sodium nuclei (^{23}Na) is also of great potential. These experiments are important since curing with sodium salts is an extensively used, ancient preservation method. Too much salt and heterogeneous salt distribution brings about health concerns. Therefore, quantitation of salt content in cured fis and related products is essential and requires tedious laboratory efforts using chemical methods. By taking sodium MR images of fis fillet it is possible to visualize the salt content of intact

(a)   (b)   (c)
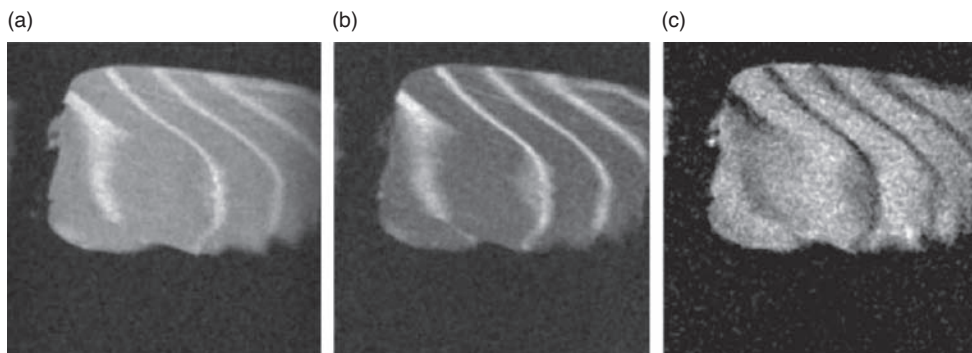
Fig. 15.3 Proton density (a), fat (b), and water (c) MR images of salmon fillet. In the proton density image, protons from both water and fat are visible, whereas in fat (b) and water (c) images, only protons from fat and water are detected, respectively. Adapted from Veliyulin *et al.* [5], with kind permission of Springer Science and Business Media.

tissues [55]. However, possibilities for quantificatio studies by ^{23}Na MRI is hampered by a phenomenon named as NMR invisibility of sodium nuclei [56,57].

Although MRI studies have the potential to become absolutely adaptable to different problems in the fis industry, the relatively high investment and maintenance costs of the infrastructure, as well as the required skilled personnel reduces the extent of its application areas. Another drawback limiting the use of MRI only for research purposes is that on-line production site MRI applications could not satisfy the speed requirements of the industry.

15.5   Conclusions

Quality and health concerns originating from the consumers of modern times necessitate fast, accurate, easy-to-employ, and environmentally friendly methods of testing fis and related products. The scientifi community within the fiel has been developing and serving various methods over the years, no matter whether these are traditional or unfavourable ones. NMR spectroscopy, right on this point, discloses almost an endless area of possibilities to be explored by scientists. As attempts to provide some highlights of NMR applications for fis and related products are made in this chapter, we have, together, seen that all of the LF, HF, and MRI methods have the potential to answer many questions concerning fis and related products (Table 15.1). Among these, the cross-thematic approaches within the fiel seem to be the most promising ones in order to fin the optimal solutions to the raised issues. As it is true for other field of applied sciences, NMR specialists, food scientists, biologists, chemists, and other associated people must work together in order to overcome the barriers laid by the traditional educations and stiff principles.

It is obvious that NMR applications in both fis industry and research efforts will be replacing the traditional techniques progressively. The rate of this replacement and enlarging the horizon of new NMR applications within the fiel will certainly be boosted by the advancements (speed, sensitivity, and ease of use, etc.) and availability of the NMR instruments and developing strong awareness within the scientifi and industrial community.

Table 15.1 Various application areas of different NMR methods on quality assessment of fish and related products

Method	Area of application	Subject	Reference
LF-NMR	Fat and water content, water holding capacity	Salmon and cod	[13]
	Effects of frozen storage	Cod	[12]
	Effects of chilled and frozen storage	Cod	[22]
	Texture changes in frozen fish flesh	Cod	[24]
	Water distribution	Cod	[11]
		Herring	[23]
	Effect of salting-desalting on water holding capacity	Cod	[10]
		Cod and salmon	[58]
	Quantification of lipids	Salmon	[9,16]
		Herring	[14]
	Non-destructive measurements of fat content	Live salmon	[18]
	Simultaneous determination of protein, fat, and moisture	Fish feed and raw ingredients	[3]
HF-NMR	Quantification of betaine in mussels	Mussels	[28]
	Quantification of n-3 fatty acids, anserine, and lactate	Salmon and fish oils	[48,59]
	Study of changes in bioactive components	Cod	[29]
	Low molecular weight metabolites	Cod and haddock	[31]
	Post-mortem metabolic changes in muscle by ^{31}P HF-NMR	Carp and oyster	[60,61]
	Quality assessment during ice storage	Halibut	[32]
	Marine lipids and phospholipids	Cod roe and milt	[49]
	Lipid deteriorations	Cod	[33]
	Determinations of n-3 fatty acids and docosahexaenoic acid (DHA)	Fish oils	[34,35]
	Omega-3 fatty acid content by HR-MAS	Salmon	[62]
	Metabolic condition changes upon thermal stress	Steelhead trout	[37]
	Metabolic response to long-term handling	Salmon	[38]
	Metabolic differences between healthy and hepatic tumour tissues	Flatfish	[39]
	Metabolic markers upon toxic exposure	Minnow, Chinook salmon, medeka, and rainbow trout	[40–43]
	Authenticity	Wild-farmed salmon fish oils	[2,47]
MRI	Backbone deformations	Salmon	[5]
	Belly bursting	Herring	[52]
	Salt content by ^{1}H and [23]Na MRI	Salmon and cod	[57]

Abbreviations: LF-NMR, low-field nuclear magnetic resonance; HF-NMR, high-field NMR; MRI, magnetic resonance imaging; HR-MAS, high resolution-magic angle spinning.

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Part II

Seafood safety

16 Food-borne pathogens in seafood and their control

Dominic Kasujja Bagenda and Koji Yamazaki

16.1 Introduction

Globalization and increasing demand for declining fisherie resources have caused an extension of seafood distribution chains. Today, seafood is being consumed further away from where it is harvested. This globalization of the seafood market has serious food safety implications. Seafood handlers must continually invest resources in the effective control of pathogens in order to protect consumers. A pathogen is a biological agent that causes disease or illness to its host. Pathogenic agents include bacteria, viruses, protozoa, fungi, parasites, and proteins. According to several studies [1–4], pathogens associated with consumption of seafood include bacteria of the species *Vibrio*, *Clostridium*, and *Listeria,* as well as pathogenic viruses, notably *Norovirus*. Furthermore, a form of illness called histamine poisoning is commonly associated with consumption of seafood. Several physical, chemical, and biological methods to control each of these pathogens have been developed.

Based on function, Gould [5] has categorized the methods used to control pathogens into those that:

- prevent access pathogens to the food;
- inactivate pathogens that access the food; and
- prevent or slow down growth of pathogens that have accessed the food and escaped inactivation processes.

A few examples of methods from each category will help in illustrating this point. Preventing access of pathogens can be accomplished by aseptic packaging of heat processed foods. On the other hand, inactivation of pathogens that have accessed the food can be carried out by heat pasteurization and sterilization, ionizing irradiation, addition of enzymes, use of high hydrostatic pressure (HHP), or electric shock treatments. Procedures to slow down or prevent growth of pathogens include chilling and freezing, curing, drying, acidifying, fermenting, vacuum and modifie atmosphere packaging, addition of preservatives, or microstructure control in water-in-oil emulsions. It is often necessary to use a set of these methods in order to completely control pathogens in seafood. In this case, each of the methods used can be thought of as a hurdle that pathogens have to overcome in order to survive until the seafood is

consumed. The combination of sets of methods to control or eliminate food-borne pathogens is commonly known as hurdle technology. The concept of hurdle technology is dealt with in subsequent sections of this chapter.

It must be emphasized that methods outlined in this chapter are neither substitutes for good sanitation, nor agents for improvement of partially spoiled seafood. Rather, these methods are simply adjuncts to good sanitation and hygiene, which take top priority in the control of pathogens in seafood. When considering good sanitation and hygiene for seafood, it is important to be aware of the risks and the pathogens that cause them. This chapter attempts to address the potential and challenges of currently available methods for controlling food-borne pathogens in seafood.

16.2   Major food-borne pathogens related to seafood

Genus *Vibrio* contains bacterial pathogens associated with seafood. According to *Bergy's Manual of Systematic Bacteriology* [6], bacteria of this genus are gram-negative bacilli or rods, with comma-shaped cells. *Vibrio* spp. are oxidase positive, facultatively anaerobic, and do not form spores. The disease-causing strains are associated with gastroenteritis, but can also infect open wounds or cause septicaemia. Notable food pathogens from *Vibrio* spp. include *V. parahaemolyticus* (associated with undercooked seafood), *V. vulnificu* (another seafood-associated, food poisoning strain), and *V. cholerae* (the causative agent of cholera). Symptoms of poisoning by *Vibrio* spp. may include watery diarrhea, nausea, vomiting, abdominal cramps, and fever. Todd [1] reports that *V. vulnificu* is a major bacterial cause of mortality associated with food-borne diseases, and results in the highest death rate of any causative agent.

Clostridium botulinum is an anaerobic, gram-positive, spore-forming rod that produces a potent neurotoxin. This neurotoxin, referred to as the botulinum toxin, is the most toxic naturally occurring substance known. The spores of *C. botulinum* are heat-resistant and can survive in foods that are incorrectly or minimally processed. According to *Bergy's Manual of Systematic Bacteriology* [6], seven types (A–G) of botulism are recognized, based on the antigenic specificit of the toxin produced by each strain. Types A, B, E, and F cause human botulism. The incidence of disease is low, but mortality rates can be high if not treated immediately and properly. Thomas *et al*. [7] reports that outbreaks are usually associated with inadequately processed, home-canned (or fermented) foods, but occasionally commercially produced foods have been involved in outbreaks.

Listeria monocytogenes is a gram-positive bacillus known to cause listeriosis. *L. monocytogenes* is resilient, surviving extended refrigeration to cause outbreaks in refrigerated foods. Several studies [8–10] report the presence of *L. monocytogenes* in seafood. Klima and Montville [11] further report that the US Food and Drug Administration (FDA) has set a zero tolerance level for *L. monocytogenes* in ready-to-eat seafood, resulting in an incidence of product recalls for cans of Canadian frozen shucked lobster. *L. monocytogenes* is of particular concern to the food industry, because it can resist heat, salt, nitrite, and acids much better than many other pathogens. Symptoms of listeriosis include septicaemia, meningitis, encephalitis, and intrauterine or cervical infections in pregnant women, which may result in spontaneous abortion or stillbirth. Signs of infection start with influenza-li e symptoms including persistent fever. Gastrointestinal symptoms such as nausea, vomiting, and diarrhoea may occur in more serious cases of listeriosis.

Histamine poisoning is caused by the ingestion of foods that contain high levels of histamine and possibly other vasoactive amines and compounds. Lehane and Olley [4] report that these compounds are formed by the growth of certain bacteria (usually members of family Enterobacteriaceae) and the subsequent action of their decarboxylase enzymes on histidine and other amino acids in fisher products, particularly tuna. However, any food that contains the appropriate amino acids and is subjected to certain bacterial contamination and growth may lead to histamine poisoning when ingested. Symptoms of histamine poisoning may include a tingling or burning sensation in the mouth, a rash on the upper body and a drop in blood pressure, nausea, vomiting, and diarrhoea. Fishery products that have been implicated in histamine poisoning include tunas (e.g. skipjack and yellow fin) bluefish sardines, anchovies, mackerel, amberjack, and abalone. According to Lehane and Olley [4], histamine poisoning remains one of the most common forms of fis poisoning in the US. In Japan, 4122 cases of scombroid poisoning were reported by the Ministry of Health between 1970 and 1980. Taylor [3] reports that the largest outbreak yet recorded in the world, involving 2656 cases. occurred in 1973 in Japan, resulting from consumption of dried horse mackerel.

Noroviruses *(Norwalk viruses)* are a family of unclassifie small round structured viruses (SRSVs). The family consists of several serologically distinct groups of viruses that have been named after the places where the outbreaks occurred. *Norovirus* infections are characterized by nausea, vomiting, diarrhoea, and abdominal pain. Headache and low-grade fever may occur. Water is the most common source of outbreaks and may include water from municipal supplies, wells, recreational lakes, swimming pools, and water stored aboard cruise ships. Shellfis and salad ingredients are the foods most often implicated in *Norovirus* outbreaks. Ingestion of raw or insufficientl steamed clams and oysters poses a high risk for infection with Noroviruses. According to Graham *et al*. [12], not all cases of Norovirus infections are symptomatic. The risk of asymptomatic food handlers unknowingly contaminating seafood with Noroviruses is considerable.

16.3   Current trends in control of seafood-borne pathogens

In ensuring safety of seafood products, handlers have a wide range of methods to choose from. A key consideration in selecting an appropriate method is its effect on acceptability and nutritive quality of the seafood product. Generally, methods that provide maximum pathogen elimination with minimum effect to the appearance, odour, and texture of the fina product are considered superior. For purpose of clarity, the methods described here are divided into biological, chemical, or physical methods. Biological methods refer to those that employ living organisms or agents derived from them. Chemical and physical methods are those that depend on chemicals such as chlorine, or physical parameters such as pressure, to eliminate pathogens. Examples of recent applications related to control of pathogens in seafood are provided under each section. Since very few methods are effective on their own, most applications involve combinations of more than one method.

16.3.1   Biological methods of controlling pathogens in seafood

Living organisms possess numerous defensive or aggressive antimicrobial mechanisms. These mechanisms have evolved for purposes of ensuring success in increasingly competitive ecosystems and can be exploited for the control of food-borne pathogens in seafood.

Table 16.1 Examples of plant metabolites effective against food borne pathogens related to seafood

Sensitive pathogen	Phytochemical	Concentration	Reference
Clostridium botulinum	Thymol	200 µg/mL	[35]
	Gallic acid	400 µg/mL	[35]
Listeria monocytogenes	Mint	42 mg/mL	[36]
	Thyme	17.5 mg/mL	[36]
	Garlic	81.3 mg/mL	[36]
Vibrio parahaemolyticus	Thyme	1000 ppm	[37]
	Bay leaf	5000 ppm	[37]
	Mint	6000 ppm	[37]

Generally, biological agents currently used to control seafood-borne pathogens originate from plants or bacteria.

Antimicrobial agents of plant origin are thought to play a role in defending the plant against pests and pathogens. These agents include alkaloids, fl vonoids, isofl vonoids, tannins, cummarins, glycosides, terpenes, and phenolic compounds, etc. Some of these agents have favourable fl vours that make plants valuable as spices and herbs. According to several reports [13–17], plant metabolites are thought to cause their antimicrobial effect by cell membrane perturbation leading to cell dysfunction. Some metabolites such as phenols may interfere with germination enzymes, and L-alanine utilization in a manner that affects germination of spores of pathogenic bacteria. Examples of plant metabolites that control growth of pathogens in food include the use of allyl isothiocyanate (AITC) from the Japanese horseradish (*wasabi*). AITC impregnated labels are currently used to prevent growth of *Vibrio* spp and other micro-organisms in raw seafood lunch boxes all over Japan. Other examples of useful antimicrobial agents of plant origin are listed in Table 16.1. According to Nychas [18], the major limitation of phytochemical compounds in the control of seafood-borne pathogens is their adverse effect on sensory aspects of food. The effective antimicrobial doses for many phytochemicals are well above organoleptically acceptable levels. Nevertheless, in combination with other antimicrobial barriers, even moderate doses of spices can enhance microbial safety of food.

Bacteria have also been used to control pathogens in food for a long time. Several studies [2,19–24] have shown that whole cultures as well as peptides and/or metabolites are very useful in the inhibition of pathogenic organisms. Among these, probiotics, nisin, pediocin, reuterin, and Sakacin continue to receive considerable research attention. Through acidifi cation and production of helpful metabolites such as alcohols, *L. planatrum* and other lactic acid bacteria play an important role in the control of pathogens during seafood fermentation. Nisin, produced by *Lactococcus lactis* subsp. *lactis*, is effective against *L. monocytogenes* and *C. botulinum*. Nisin was the firs bacteriocin to be used on a commercial scale in the food industry. It eliminates or inhibits the psychotrophic seafood pathogen *L. monocytogenes* in fresh and lightly preserved seafood products. Cold smoked salmon for example is usually vacuum packed with a salt content below 6% and a pH above 5.0. It is not heated before consumption and so the risk of *L. monocytogenes* is high. However, in a CO_2 atmosphere, nisin strongly inhibits *L. monocytogenes* in cold smoked salmon. Furthermore, toxin produc-tion by *C. botulinum* type E spores in smoked mackerel fillet stored at 10 and 26°C can be delayed by spraying with nisin before packing in a 100% CO_2 atmosphere. Occurrence of resistant variants and inefficien y against spores is a challenge for the application of nisin.

16.3.2   Physical and chemical methods of controlling pathogens in seafood

Numerous chemical and physical methods of controlling food-borne pathogens in seafood are currently in use. Examples of chemical methods include the use of chlorine related compounds and acids. Physical methods include the use of irradiation and HHP to eliminate or control pathogens. Some of these methods such as salting have been effectively used by man for a long time. The more technologically advanced methods such as irradiation are recent developments. The effective concentrations, doses, or conditions for each method vary with the type of seafood product and the pathogen that is being targeted. Though technical details of each of these methods are beyond the scope of this chapter, a quick reference list of some applications is provided in Table 16.2. Furthermore, a few examples are briefl discussed below.

Chlorine and its related compounds (chloro-cides) have strong antimicrobial properties and have been extensively used for food safety. Sodium chloride (NaCl) is one of the most common antimicrobial agents in use. Apart from being cheap and relatively safe to handle, chloro-cides have high antimicrobial effica y and low toxicity to humans. Chloro-cides are therefore widely used to clean food contact surfaces and raw material in the food industry. Chloro-cides such as sodium hypochlorite can also be easily generated by electrolyzing dilute salt solutions (0.1% NaCl) using an anode and a cathode. Sodium hypochlorite solutions generated from this process are referred to as electrolyzed water. Ren and Su [25] report that electrolyzed water exhibits strong antimicrobial action against *V. parahaemolyticus* and *V. vulnificu* . Exposing oysters to electrolyzed water (30 ppm chlorine) for 8 hours significantly decreased counts of both *V. parahaemolyticus* and *V. vulnificu* . The antimicrobial mechanism of chloro-cides has not been fully understood. Haas and Endelbrecht [26] suggest that chlorine affects the cell membrane and cellular DNA resulting in cell death. It is widely accepted that the amount of free chlorine available and the contact time are principle factors in determining the antimicrobial effectiveness of chloro-cides.

Acid antimicrobials are also useful agents for controlling pathogenic bacteria in seafood. Acid antimicrobials currently in use include lactic acid, acetic acid, and citric acid. Sun and Oliver [27] reported that lactic acid (300 ppm) inhibits *V. vulnificu* , a pathogen associated with consumption of raw oyster. Another acid antimicrobial, acetic acid, is widely used to control food-borne pathogens. According to two reports [28,29], acetic acid and salt combine well to prevent outgrowth of *C. botulinum*, during the fermentation of sushi in Northern Japan. Acetic acid is cheap, widely available, and generally accepted well by consumers. It therefore has high potential for use in the control of food-borne pathogens in seafood. Citric acid is similar to but weaker than acetic acid. Citric acid has a pleasant sour taste and, according to Post *et al*. [30], has been successfully used to control *C. botulinum* growth and toxin production in shrimp puree.

A good example of a physical method for controlling pathogens in seafood is irradiation. Irradiation is the process of exposing food to ionizing radiation in order to achieve sterility. It is sometimes referred to as cold pasteurization to avoid consumer fear "radiation", and emphasize its similarities to the process of pasteurization. Harewood *et al*. [31] reports that viral pathogens in hard shelled clams can be inactivated using 26.3 kGy of gamma irradiation.

HHP is another physical method for controlling pathogens in seafood. According to Hoover *et al*. [32], HHP kills or sub-lethally injures cells by disruption of the cell wall and membrane, dissociation of protein and ribosomal subunit structures, and loss of activity of

Table 16.2 Examples of physical and chemical methods effective against food borne pathogens related to seafood

Target pathogen	Technique	Seafood	Notes	Reference
Listeria monocytogenes	Trisodium phosphate	Trout fillets	Fillets dipped in 20% trisodium phosphate	[38]
	Sodium acetate	Crab meat	Washing in 4 M sodium acetate	[39]
	Sodium lactate (2.4%) and sodium diacetate (0.125%)	Smoked salmon fillets	Applied to the surface of fillets and stored at 4°C	[40]
	Pulsed UV light	Raw salmon fillets	Exposure to 5.6 J/cm^2 (3 pulses/sec) for 60 s at a distance of 8 cm	[41]
	Electrolyzed oxidizing water	Raw salmon	Dipped fillets in acidic electrolyzed water (2 min 22°C, 50 ppm chlorine)	[42]
	Aqueous chlorine dioxide	Fish cubes (*Lutjanus griseus*)	Dipping in 100 ppm ClO$_2$ solution for 5 min	[43]
	Salt, smoke (phenol), and high pressure	Dolphinfish (*Coryphaena hippurus*) fillets	1.97% salt, 42 ppm phenol, and high pressure treatment of 300 MPa at 20°C for 15 min	[44]
	Vacuum packaging and mild heat pasteurization	Rainbow trout roe	Mild heat; 62°C, 15 min	[45]
	Brine, potassium lactate (2.1%), sodium diacetate (0.12%)	Cold smoked salmon fillets	Fillets were injected with the brine solution	[46]
Vibrio spp	HHP	Oysters	$\geq$350 MPa for 2 min at 1 to 35°C HHP	[33]
	Mild heat		Mild heat; 50°C, 10 min	[47]
	Electrolyzed water		Hold in electrolyzed water (30 ppm chlorine) for 8 h	[25]
Clostridium botulinum	Acetic acid and salt	Fermented salmon	Added during fermentation	[28]
	Citric acid	Shrimp puree	Final pH 4.2	[30]
Viral pathogens	Gamma irradiation	Hard shelled clams	26.3 kGy	[31]
Pathogenic bacteria (general)	Acidified sodium chlorite	Finfish and crustaceans	Dip/spray of 1200 ppm, pH 2.3–2.9. Cooked before consumption	[48]

some enzymes. Kural *et al*. [33] report that HHP (up to 350 MPa for 2 minutes at 1–35°C) can be used to inactivate *Vibrio* spp in oysters.

16.3.3   Hurdle technology for controlling pathogens in seafood

To ensure elimination or control of pathogens in seafood, a combination of several factors is usually needed. These factors may be thought of as hurdles that the pathogen has to overcome in order to be present in the seafood product. An intelligent combination of hurdles would therefore ensure that all pathogens are eliminated or inactivated in the fina seafood product. Based on the hurdle effect, a concept referred to as hurdle technology has been developed. Leistner [34] define hurdle technology as the intelligent combination of hurdles to secure safety, stability, sensory, nutritive, and economic aspects of a food product. Hurdles in a food system may be composed of factors such as high temperature during processing, low temperature during storage, water activity, acidity, redox potential of the product, as well as preservatives. Depending on the risk and the type of pathogen, the intensity of the various hurdles may be adjusted to suit consumer tastes and economic regimes without sacrificin safety aspects of the product. Traditionally, fermented seafood products common in Japan, provide a typical example of hurdle technology. Fermentation of sushi employs hurdles that favour growth of desirable bacteria but inhibit the growth of pathogens. Important hurdles in the early stages of fermentation are salt and vinegar. Raw fis is cured in salt (20–30%, w/w) for one month before being desalted and pickled in vinegar. The main target of these hurdles is *C. botulinum.* Growth of lactic acid bacteria during fermentation results in acid production from metabolism of added sugars and rice. The result is a pH hurdle important in controlling growth of *C. botulinum*. According to Sasaki *et al*. [28], the pH of fis drops from 6.4 to 4.9 during the ripening process. Other examples of hurdle technology are included in Table 16.2.

16.4   Conclusions

World fisherie trade continues to grow as seafood consumption booms globally. To facilitate global trade, seafood is being transported further, requiring extended storage and increased handling. Increased storage time and handling expose seafood to higher risks of contamination. Currently, several food-borne disease outbreaks are associated with consumption of ill-prepared or ill-handled seafood. Notable outbreaks include those caused by *Vibrio* spp, *C. botulinum*, *L. monocytogenes*, histamine (scombroid) poisoning, and Noroviruses (Norwalk viruses). Even though most of these pathogens can be controlled by physical, biological, or chemical means, care must be taken to preserve the nutritive and economic aspects of the seafood products. Consequently, application of a single method at lethal doses is being replaced by application of several methods at sub-lethal doses. This trend towards combination of methods is the basis of hurdle technology. For successful implementation of hurdle technology, it is important to understand the effects of each of the hurdles on the target pathogens, as well as how best the hurdles can be combined to maximize sensory, nutritional, and economic value without compromising the safety of the seafood product.

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17 Novel approaches in seafood preservation techniques

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17.1 Introduction

Seafood plays an important role in human diet and is considered not only a reliable source of protein, but also of nutritional significanc due to its lipid, vitamin, and mineral constituents. Thus, these constituents are important in consumers' interest in fis products due to the nutritional value and health-promoting characteristics of seafood. However, seafoods are perishable products and the shelf-life is limited in the presence of air and atmospheric oxygen (O_2), which also lead to the growth of aerobic spoilage micro-organisms. Hence, preservation of the fresh quality of seafood is essential. Preservation techniques are designed to inhibit or reduce the metabolic changes that lead to fis quality deterioration. Many different techniques have been used to prolong the shelf-life of seafoods and these techniques are based on the control of temperature, water activity, microbial loads, and the available oxygen. This chapter focuses on novel preservation techniques in seafoods.

17.2 Seafood preservation techniques

17.2.1 Modified atmosphere packaging (MAP)

MAP is a form of packaging involving the removal of air from the pack and its replacement with a single gas or a mixture of gases. MAP has become an increasingly popular preservation technique in seafood distribution and marketing to meet consumer demands. MAP techniques are now used in a wide range of applications in food products, including raw and cooked red meats, poultry, fruit, fresh pasta, crisps, coffee, tea, vegetables, cheese, bread, fish and crustaceans, etc. [1].

MAP, together with refrigeration, is capable of extending the shelf-life of fis and shellfish Modificatio of the atmosphere within the package by decreasing the oxygen concentration while increasing the content of carbon dioxide (CO_2) and/or nitrogen (N_2) has been shown to significantl prolong the shelf-life of perishable food products at chill temperatures. However, undoubtedly the single most important concern with the use of MAP products is the potential for the outgrowth and toxin production by the non-proteolytic, *Clostridium botulinum* type

E, which can grow at low temperatures. There is a change in the composition of microflor in the MAP products, which might also contribute to these risks [2,3]. The shelf-life of fis products in MAP can be extended greatly, depending on raw materials, temperature, gas mixture and proportion, gas/product ratio, and packaging materials, etc.

The main gases used commercially for MAP are CO_2, oxygen (O_2), and N_2, although trace gases have been suggested, including carbon monoxide, nitrous and nitric oxides, sulphur dioxide, ethane, and chlorine. Apart from these, noble gases such as xenon, argon, and helium are of increasing interest for MAP systems. Argon and helium have been permitted as food grade gases by EC legislation since 1996 [4]. However, most of these trace gases have not been developed due to concerns about safety, consumer response, legal aspects, and cost [5]. CO_2, O_2, and N_2 are used in different combinations and ratios depending on the product and the needs of the manufacturer and the consumer [4]. The effectiveness of MAP on different fis species has been investigated [6,7]. The recommended gas mixes for retail packing are $CO_2/N_2/O_2$ (40:30:30) for white fis and shellfis and CO_2/N_2 (60:40) for fatty and smoked fis [8]. The gas/products ratio differs from 2:1 to 5:1 for modifie atmosphere packed fish while the CO_2 concentration varies between 20 and 100% [9]. The UK Sea Fish Authority recommends that a gas to product ratio should be 3:1, with a minimum concentration of 20% CO_2, in order to render a noticeable benefi in storage life of fish

17.2.2   Irradiation technology

Food irradiation is an old-food preservation technique. Its commercial use began in 1957 in Germany to improve quality of spices [10], while the US Food and Drug Administration (FDA) irradiated some food items in 1970 [11]. This method is approved by the EU and the US authorities only for a limited number of foods or food products using a limited range of doses [12–14]. In 2005, the total quantity of irradiated foods in the world was reported to be about 405,000 tons, while meat and seafood consisted of 8% of that amount (32,400 tons) [15].

Irradiation of food products is a physical treatment involving direct exposure to electron or electromagnetic rays, to preserve food for a longer time and improve their safety and quality [16]. For food preservation, gamma rays, X-rays, and accelerated electron beams are used as sources of ionizing radiation. Among the gamma-ray emitting material, cobalt 60 and caesium 137 are used, and cobalt 60 is more important for food irradiation systems [17]. However, there are some disadvantages when radiation is used (Table 17.1).

Irradiation with high-energy beams leads to the breakdown of chemical bonds and formation of free radicals and ions. Breakdown of sufficien numbers of critical bonds in organisms in the food leads to their destruction [18]. Although little is known about the mechanisms of bacterial inactivation by irradiation, it is reported that irradiation inactivates micro-organisms mainly by causing lesions in the DNA [19]. The required dose to reduce the number of viable specifi nonspore-forming pathogenic bacteria in food is in the range of 2 to 8 kGy, while 0.4 to 10 kGy doses are necessary for quality preservation of food by causing a substantial decrease in the number of viable specifi spoilage micro-organisms [20]. Low-dose irradiation is effective for fis preservation when used in combination with other processes such as chilling or heating [21]. Irradiation doses of 1.0 to 2.0 kGy effectively eliminate contaminant micro-organism [22] in raw fis and seafood dishes such as sushi, fresh live oyster, and clams [23]. The UK Food Irradiation Legislation (SI, 1990:2489) permits the use of 3 kGy radiation for fis and shellfis [24].

Table 17.1   Disadvantages of some nonthermal techniques

Irradiation [58][a] [107]	Pulsed electric field [41,58]	High hydrostatic pressure [58]	Ultrasound [41,58]	Pulsed intense light [98]
Low energy and intensity, continuous emission for Cobalt 60	High cost equipment	High cost equipment	Maximum amplitude of industrial-scale transducers limited	Only applied for clean surfaces
Licensing tightly regulated and consumer concerns for Caesium 137	Small capacity of treatment chamber	Increased metal fatigue	High energy consumption	Only works on exposed bacteria
Limited penetration and potential for interruption for electron beam	Electrode erosion	Long cycle times	Long treatments times	Cause severely blindness for workers at high dose
Low conversion efficiency and high heat in converter plate for X-ray	Safety concerns in local processing environment	Undesirable sensory changes	Complex mode of action	Limited use in opaque food-stuff
Maximum intensity limited to dose of 10 kGy	Regulatory issues remain to be resolved		Needs to be used in combination with another process (e.g. heating)	Undesirable sensory changes
Undesirable sensory changes	Undesirable sensory changes		Undesirable sensory changes	

[a]These limitation of nonthermal technologies occur at high intensities.

17.2.3   Ozone (O₃) preservation technique

The history of ozone and its application has been extensively reviewed [25,26]. Ozone is formed in the Earth's atmosphere as a result of lightening or high-energy ultraviolet radiation. Ozonation is a relatively new method for food processing and has been used safely and effectively in water treatment. Water treated with ozone reduces the bacteria on the contact surfaces where foods are processed, so can be used for cleaning and sterilization of processing plants. In addition, ozone does not produce significan toxic residues in the environment after the treatment [27].

Recently, the effects of ozone treatment on the sensory quality and shelf-life of fis have been investigated [28–30]. The use of ozone in shelf-life extension of fresh fis is a promising food preservation technology [27,31]. Molecular ozone and its decomposition products are effective in destroying micro-organisms, including bacteria, viruses, and fungi, due to their effects on microbial intracellular enzymes, nucleic acids, and other cell components [30,32]. Campos *et al*. [30,33] reported that the combined use of ozone and slurry ice produced an additional reduction in the counts of the anaerobes, psychrotrophic bacteria, and of both proteolytic and lipolytic micro-organisms during storage of fish

Ozone reduces the superficia contamination of the fis as well as the formation of volatile N_2 compounds, thus improving the sensory quality for several days when it is stored under refrigeration [34]. The water treatment with ozone seems to have a selective quality, especially with respect to reducing trimethylamine-oxide (TMA-O) micro-organisms, an effect

observed mainly during the firs six days of storage of the fis kept at refrigeration temperature [28]. The effectiveness of ozone pretreatment on fresh muscle of tilapia has been studied [35]. An increase in the total volatile base-N_2 (TVB-N) was observed after 8 and 22 days of storage, at 5 and 0°C, respectively, but differences between control and treated samples were not significan ($P > 0.05$) at both temperatures. On the other hand, a clear effect on the bactericidal action of ozone in fis at 0°C was observed, but not at 5°C. The effect of ozone on shucked mussels [36] and vacuum-packaged rainbow trout [31] have also been studied at 4°C. After submerging for 90 minutes in ozonized water (1 mg/L), low TVB-N content was observed and the product had a shelf-life of 12 days compared with 6 days for the non-ozonized samples.

17.2.4 **Physical preservation methods**

17.2.4.1 *Pulsed electric fields (PEF)*

PEF is a nonthermal food processing technique that has received increased interest over the last few years [37]. The method is based on the use of electric field to eradicate food-borne pathogens and to control spoilage micro-organisms in foods. Therefore, it is likely that the initial application of PEF preservation will be aimed at replacing thermal pasteurization of foods [38]. This process is mainly used in liquid foods [37,39]. It preserves quality attributes such as sensory quality and nutritional value, as well as controlling the microbiological safety of products [40]. PEF suffers from some disadvantages such as being ineffective on enzymes and spores, adverse effects on foods, and certain safety concerns in local processing environments [41]. The lethal effect of PEF treatment is dependent on the electric fiel strength and treatment time and bacterial features [42,43]. Gudmundsson and Hafsteinsson [44] reported that PEF treatment of meat and fis was not suitable for preservation because it affected the texture and microstructure at lower fiel voltage than effectively reducing bacterial growth.

17.2.4.2 *Ultraviolet (UV) radiation*

UV irradiation is nonthermal and environmentally friendly. This process includes the exposure of the product to a germicidal light with a wavelength to inactivate bacteria and viruses [45]. UV radiation consists of three classes according to wavelength:

1) UV-A, also known as near-UV, ranges from 315 to 400 nm;
2) UV-B, mid-range UV, ranges from 280 to 315 nm;
3) UV-C, far-UV, ranges rom 100 to 280 nm [46].

UV-C is a more effective biocide than UV-A and UV-B [47], because most micro-organisms absorb UV light at a 254-nm wavelength, which is sufficien to cause physical shifting of electrons and breaking of bonds in the deoxyribonucleic acid (DNA), preventing life and reproduction [48]. UV-C light treatment has been used in the food industry for different purposes, including sterilizing of food contact surfaces, of water used for rinsing of food, air sanitation in the meat and vegetable processing, and reduction of pathogen micro-organisms in fruit, vegetable, red meat, poultry, and fis processing [49–53]. Generally, the resistance to UV irradiation is shown to follow the trend: Gram-negative < Gram-positive < yeast < bacterial spores < moulds < viruses [48]. Although, UV radiation treatment improves the

nutritional value of some foods [54,55], negative effects have been reported on chemical and sensory characteristics such as rancidity, tallowiness, fishiness cardboard fl vour, and oxidized fl vour of oil and food [56,57].

17.2.4.3   Oscillatory magnetic fields (OMF)

OMF are nonthermal physical processes [58], which receive considerable attention, since in combination with conventional preservation techniques they are of potential use for the microbial stabilization of fresh-like food products with little induced degradation of sensory and nutritional properties [59]. Research on the biological effects of magnetic field dates back to as early as 1938 [60]. However, the use of magnetic field as a nonthermal technology for food preservation was firs proposed in 1985, when a US patent was granted to Hofmann [61]. In the OMF technique, food is sealed in a plastic bag and subjected to 1 to 100 pulses in an OMF with a frequency between 5 and 500 kHz at 0 to 50°C for a total exposure time of 25 to 100 μs [62,63]. In the patent application a frequency of 5 to 50 kH, and intensity of 5 to 50 Tesla was specifie for preservation [64]. However, there is a significan lack of information on assessing the potential of this technology in foods and seafood. Lipiec *et al*. [65] reported that OMF might be used for disinfecting agricultural products and food.

17.2.4.4   High pressure processing (HPP)

HPP is based on the application of very high hydrostatic pressures ranging from 100 to over 1000 MPa at temperatures of 0 to 100°C, with varying holding times to process different foods [66]. The application of HPP has been extensively reviewed [67,68]. The effect of HPP is variable and dependent on not only the operating conditions, but also food composition. This technique allows inactivation of enzymes and micro-organisms, and has minimal effects on the quality of foods [69]. HP-processed foods were firs commercialized in Japan in 1992. Following initial successes with fruit juices and jams, the technology is now applied to an increasing range of food products, including smoothies, ham, guacamole, salsa, rice products, fish and shellfis [68].

   The major advantage of HPP technology is the production of safer foods that retain the appearance, fl vour, texture, and nutritional qualities of the unprocessed product from the consumer's point of view [69,70]. This is because, in contrast to heat, HPP does not disrupt covalent bonds and the primary structure of proteins remains relatively unaffected [67]. Many studies have demonstrated that pressure in the range of 300 to 600 MPa can inactivate many fungi and vegetative bacteria [71]. Fish and shellfis are generally spoiled by Gram negative bacteria [72]. Seafood treated with HPP has higher proportions of Gram-positive bacteria due to the greater susceptibility of Gram-negative species to HPP [73,74]. Factors affect the HPP-resistance of bacteria such as strain, growth phase, and temperature [75,76]. Changes in K value and lipids in seafood subjected to high pressure have also been reported [77,78]. Application of HPP decreased inosine monophosphate (IMP) levels in carp muscle [76] and provided low K values in tilapia fillet [78]. Marshall *et al*. [79] used different doses of HPP ranging from 150 to 600 MPa for mahi mahi and tilapia in order to determine lipid oxidation changes. The study result showed that mahi mahi processing with HPP had no effect on lipid oxidation, while there was a slight increase in lipid oxidation for tilapia as pressure increased to 550 MPa. Moreover, Yagiz *et al*. [80] found that HPP of trout increased oxidation as shown by an increased level of thiobarbituric acid reactive substances (TBARS) with increased pressure. During the storage period (6 days) of mahi mahi, highest oxidation

was observed at 300 MPa. Thus HPP treatment at 300 MPa was reported to be useful for quality preservation of fis fles [81].

17.2.5   Ultrasound as a preservation technology

Ultrasound technology is a rapidly developing fiel of research, due to its increasing use by the food industry for both the analysis and modificatio of products [82]. It is a sound energy with a frequency range that covers the region from the upper limit of human hearing, which is generally considered to be 20 kHz [83]. There are two types of industrial ultrasound application, high-intensity (power) ultrasound (HIU) and low-intensity ultrasound (LIU). HIU is applied to modify a process or a product, whereas LIU is used for monitoring a process or a product [84]. HIU applications are usually found at intensities higher than 1 W/cm^2 and at frequencies between 18 and 100 kHz [85]. HIU has a wide range of commercial applications, such as emulsification homogenization, extraction, control of crystallization processes, dewatering, low temperature pasteurization, degassing, defoaming, the induction of oxidation reactions, particle size reduction and viscosity alteration, activation and inactivation of enzymes [82,86,87], biodegration of pollutants in water [88], food drying [89], meat tenderization [86,90], meat brining [91], freezing of food [86], and thawing of frozen foods [92].

Development of ultrasound technology in terms of microbial inactivation started in the 1960s, after it was discovered that the sound waves used in anti-submarine warfare killed fis [93,94]. The bactericidal effects of ultrasound result from intracellular cavitations, due to micro-mechanical shocks that disrupt the cellular, structural, and functional components, resulting in cell lysis [95]. This technology is most effective when used in combination with heat and pressure, but it can be used alone for fruit juices, sauces, purees, and dairy products [96]. Li *et al*. [97] investigated the effect of HIU (30 Hz, 800 W) on the allergenicity of shrimp allergen. The results suggested that HIU could be used to reduce the allergenicity of shrimp. Like other innovative food processing technologies, ultrasound application on seafood needs to be developed.

17.2.6   High intensity light

High intensity light is a synonymous term with pulsed UV light, pulsed light, broad spectrum white light, pulsed white light, and near infrared light; and define the range of light in which non-ionizing irradiation is emitted [98,99]. Intense light pulses is an alternative preservation technique to thermal treatment for killing micro-organisms using short-time high frequency pulses of an intense broad spectrum, rich in UV-C light [100–103], which is the portion of the electromagnetic spectrum corresponding to the band between 200 and 280 nm. Pulsed light is produced using technologies that multiply the power manyfold [99]. This method can be used for surface sterilization of foods such as baked goods, seafood and meats, fruits, and vegetables. Morris *et al*. [96] indicated that pulsed high-intensity light showed some promise in packaged material and product microbiological destruction. Rowan *et al*. [104] reported food related micro-organism such as *Listeria monocytogenes*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus* were reduced by up to 2 or 6 log$_{10}$ cfu/mL, with 200 light pulses (pulse duration, 100 ns) of low or high UV content, respectively. Although many studies are related to dairy products, vegetables, and fruits, there are limited studies about effectiveness of pulsed light on fis microbial flor and shelf-life. Dunn *et al*. [105] reported that significan reductions in microbial population enhanced

shelf-life for shrimp treated with pulsed light. Ozer and Demirci [106] found that about one log reduction (90%) of *Escherichia coli* O157:H7 or *L. monocytogenes* on salmon fillet could be achieved with pulsed UV light treatment (5.6 J cm^2) per 60-second treatment at 8 cm distance without affecting the quality. Limitation of these technologies are given in Table 17.1.

17.3   Conclusions

Food preservation techniques extend shelf-life of products while maintaining food safety and quality. To improve the effectiveness of preservation techniques, two or more of the techniques described above can be combined and also the use of technology such as ultrasound, high intensity light, membrane filtration and pulsed electric applications on seafood need to be investigated.

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18 Essential oils: natural antimicrobials for fish preservation

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18.1 Introduction

Fish are highly susceptible to spoilage, which is caused mainly by microbial growth and metabolism that produce amines, sulphides, alcohols, aldehydes, ketones, and organic acids. Spoiled products have unpleasant and unacceptable off-fl vours, making fis that is not well protected unsuitable for human consumption. Gram-negative bacteria are mostly found in fresh fis and are responsible for fis spoilage. Gram-negative, fermentative bacteria such as *Vibrionaceae*, cause spoilage of unpreserved fish whereas psychrotolerant gram-negative bacteria *Pseudomonas* spp. and *Shewanella* spp. grow in chilled fish Meanwhile, *Photobacterium phosphoreum* and lactic acid bacteria spoil packed fish *Lactobacillus* and *Carnobacterium* associated with gram-negative fermentative bacteria, such as *P. phosphoreum* and psychrotrophic *Enterobacteriaceae*, are the dominant bacteria in lightly salted, acidified and chill-stored vacuum packed fish Spore-forming bacteria such as *Clostridium* or *Bacillus* may grow in unsalted heat-treated fis products [1–4].

Improving the safety and quality of seafood is important for both the consumers and the seafood industry. Synthetic or natural preservatives can control microbial growth and improve the safety and quality during fis storage. Consumers prefer natural preservatives to their artificia counterparts, which may potentially have undesirable effects on health. Essential oils produced from some kinds of plants, such as herbs and spices, are representative natural preservatives for fis or fis products [5–8].

This chapter introduces antibacterial properties of essential oils against fis spoilage bacteria, focusing on the active components of essential oils and their effective application for the preservation of fis and fis products.

18.2 Essential oils

18.2.1 Chemistry of essential oils

Essential oils contain many kinds of chemical compounds that have a variety of biological activities. The main components are terpenes, alcohols, hydrocarbons, phenols, aldehydes,
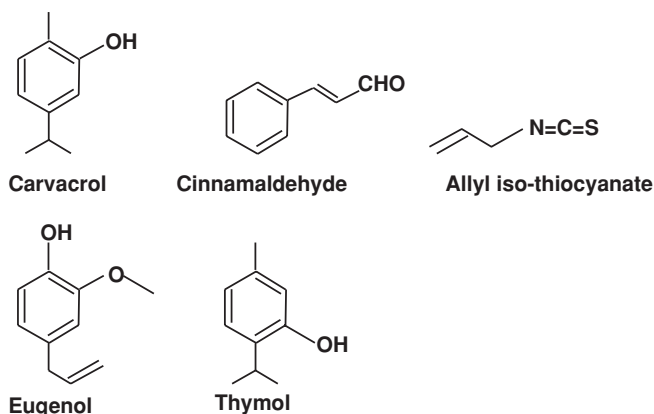
Fig. 18.1   Structures of active compound from essential oils.

esters, and ketones, which occur naturally as major components in plants. Phenols found in clove, oregano, and thyme oils, are highly antimicrobial against a wide range of micro-organisms. Terpenes (including monoterpenes, diterpenes, sesquiterpenes, etc.) are the most abundant components of the essential oils from many plants. There is no doubt that herbs and spices are the most important sources for production of essential oils.

18.2.2   Active components of essential oils

Essential oils are plant extracts from herbs and spices such as allspice, anise, basil, bay, caraway, cardamom, cinnamon, clove, coriander, cumin, eucalyptus, fennel, garlic, ginger, horseradish, lemongrass, mace, mandarin, marjoram, mint, mustard, nutmeg, onion, orange, oregano, parsley, rosemary, saffron, sage, and thyme, etc. They have different ranges of antimicrobial activities against spoilage and pathogenic bacteria [9–19]. Phenolic compounds such as carvacrol, eugenol, and thymol (Fig. 18.1) have the strongest antimicrobial properties [20–29]. Cinnamaldehyde and allyl isothiocyanate (Fig. 18.1) also show strong antimicrobial activities, as reported in some studies [30].

18.2.3   Bacterial sensitivity to essential oils and their components

Gram-positive bacteria are more sensitive to essential oils than gram-negative bacteria [31–39]. Gram-negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic components through its lipopolysaccharide constituents [8,39–41].

18.2.4   Phenolic compounds

The antimicrobial activity of essential oils cannot be explained by one specifi mecha-nism, because there are several molecular targets in the bacterial cell for active components of essential oils [39,42]. Phenolic compounds are the main group responsible for the an-timicrobial activity of essential oils. They can dissolve the hydrophobic domain (lipids)

of the bacterial membrane, disintegrate the outer membrane, increase the permeability of the cytoplasmic membrane to adenosine-5′-triphosphate (ATP), and inactivate the cytoplasmic enzymes. Antimicrobial effects of phenolic compounds are based on these biological activities [9,39,43–47].

18.3   Application of essential oils to fish preservation

Essential oils are soluble in alcohol, but poorly soluble in water. They are widely used in the food industry as fl vourings and preservative agents [48]. Fish is one of the most common food materials that are traditionally combined with many spices and herbs [9,48]. From the industrial and marketing point of view, the shelf-life of fis is very important. If the shelf-life can be prolonged by one or two days, it allows a better marketability and more profi [48]. Essential oils are relatively inexpensive compared with other preservative agents or technologies such as smoking, and irradiation, etc. However, essential oils influenc the sensory characteristic of fis and should be added at low concentrations for preserving fis [44].

Most studies have evaluated the antimicrobial effect of essential oils and/or their active compounds *in vitro*. However, few investigations have reported their potential role in fis [49–55]. In general, higher concentrations of essential oils or their active compounds are required to achieve the same antimicrobial effect in food as compared to that in culture media [26,56–58]. This is due to the interaction between active compounds of essential oils and nutrients of foods [7,10,45,55,59].

This chapter describes the effect of essential oils or their active compounds on bacterial growth in culture media and on fis preservation. The combination effect of essential oils with other preservatives is also reviewed.

18.3.1   Effect of essential oils on fish spoilage bacteria

Mahmoud *et al*. [19] reported the antimicrobial activity of garlic essential oil, allyl isothiocyanate, carvacrol, cinnamaldehyde, citral, cuminnaldehyde, eugenol, isoeugenol, linalool, and thymol against 14 bacterial strains (in culture media) isolated from carp (*Cyprinus carpio*) using the paper disc diffusion method. The diameter of the growth inhibition zone was used as the criterion for measuring the antimicrobial activity of garlic oil and its active compounds. Phenolic compounds, thymol and carvacrol, aromatic aldehyde, and cinnamaldehyde showed the strongest antimicrobial activity against the bacterial isolates from carp. These results are in agreement with those obtained by other researchers [10,21,27]. The above phenolic compounds that contain a hydroxe group and a molecular mass of 150 to 160, have been reported as the most effective antimicrobial components found in essential oils [9,56]. The combination of these phenols was also effective in the inhibition of bacterial growth [19]. The combination of carvacrol and thymol has the strongest antimicrobial activity, followed by cinnamaldehyde and thymol, and then carvacrol and cinnamaldehyde. These results indicate that a combination of two compounds has a stronger antimicrobial effect than the individual one [19,60].

Mejlholm and Dalgaard [44] evaluated the antimicrobial effect of nine essential oils (basil, bay, cinnamon, clove, lemongrass, marjoram, oregano, sage, and thyme) on the growth of a mixture of fi e strains of *P. phosphoreum*, a specifi spoilage organism responsible for spoilage of modifie atmosphere-packaged cod fillets in a liquid medium. All tested essential

oils reduce the growth rate of *P. phosphoreum*. The antimicrobial effect depends on the oil type and the concentration; essential oil concentrations of 0.005 to 0.1% (v/v) are required for inhibition of the growth of *P. phosphoreum*. Oregano and cinnamon oils have the strongest antimicrobial activity followed by lemongrass, thyme, clove, bay, marjoram, sage, and basil oils.

Prasad and Seenayya [61] studied the effect of essential oils from 20 spices at concentrations of 0.1 to 2% (w/v) against 18 strains (isolates from salt cured fish) namely 12 *Salinococcus roseus*, 5 *Halococcus turkmenicus*, and 1 *Halococcus morrhuae*. Clove powder and its essential oil were the most effective agents, even at a low concentration (0.1%). Onion, coriander, garlic, asafetida, mustard, and spilanthes showed excellent growth control, followed by red chillies, turmeric, ginger, cumin seed, and fenugreek.

Yano *et al*. [62] studied the antimicrobial effects of essential oils from 18 spices and herbs at fina concentrations of 2.5% against a food-borne pathogen bacterium, *Vibrio parahaemolyticus* (fis contaminants bacteria) incubated at different temperatures (5 and 30°C). Basil, clove, garlic, horseradish, marjoram, oregano, rosemary, and thyme exhibited good antibacterial activities against tested bacterium. The lowest minimum inhibitory concentration (MIC) was 0.125% observed in clove and marjoram at 30°C and 0.063% in marjoram and turmeric at 5°C. *V. parahaemolyticus* was more sensitive at low temperatures [63]. These results suggest that spices, herbs or their extract can be practical for protecting fis from the risk of contamination by *V. parahaemolyticus*.

18.3.2   Effect of essential oils on shelf-life of fish

Utilisation of essential oil and their active compounds to extend the shelf-life of fis has previously been reported (Table 18.1). Treatment of carp fillet with 0.5% carvacrol + 0.5% thymol solution for 15 min significantl decreased the initial total microbial count [19]. Harpaz *et al*. [46] reported that treatments with 0.05% oregano and/or thyme lead to remarkable reduction in the bacterial growth of *Pseudomonas* spp. and *Salmonella putrefaciens* (mostly responsible for the spoilage of fis during cold storage) on Asian sea bass fish Tassou *et al*. [7] studied the effect of oregano oil against inoculated *Staphylococcus aureus* and *S. enteritidis* on fis fillet under an aerobic and modifie atmosphere of 40% CO_2, 30% O_2, and 30% N_2 or air at 0°C. The treatment had a good bacteriostatic effect on both natural flor and inoculated bacteria on the fillets Tassou *et al*. [7] also reported the effect of mint essential oil (0.5, 1.0, 1.5, and 2.0%, v/w) on inoculated *S. enteritidis* and *Listeria monocytogenes* on three models of fis foods, namely tzatziki (pH 4.5), taramasalata

Table 18.1   Essential oils and essential oil compounds for fish preservation

Fish	Essential oil/compounds	Reference
Carp (*Cyprinus carpio*)	Carvacrol, thymol	[19,47]
Sea bass (*Lates calcarifer*)	Oregano, thyme	[46]
Cod (*Gadus morhua*)	Oregano	[44]
Shrimp (*Penaeus* spp.)	Thyme, cinnamon	[67]
Sea bream (*Sparus aurata*)	Oregano	[7]
Cod (*Gadus morhua*)	Mint	[7]
Mackerel muscle extracts	Cardamom, allspice, chilli, cinnamon, clove, cumin, black pepper, nutmeg, sage, thyme	[64]

(pH 5.0), and paté (pH 6.8) at 10°C for one week. The population of *S. enteritidis* was completely inhibited in tzatziki and significantl decreased in the other two fis foods. The population of *L. monocytogenes* decreased during the storage period.

Mejholm and Dalgaard [44] screened the effect of nine essential oils (basil, bay, cinnamon, clove, lemongrass, marjoram, oregano, sage, and thyme) against the spoilage fis bacteria, *P. phosphoreum in vitro*. Among all tested essential oils, oregano oil showed the best inhibition effect on bacterial growth. Treatment with oregano essential oil yielded a pleasant fl vour to cold fillet and significantl increased the shelf-life to 26 days as compared with 12 days for the control at 2°C. On the other hand, treatment with 0.05% oregano essential oil had a minor antimicrobial effect on salmon fillets while the treatment was more effective on cod fillets The difference in the antimicrobial effect of oregano essential oil on cod and salmon fillet is due to the higher lipid content of salmon fillet compared to cod fillets

Wendakoon and Sakaguchi [64] studied the effect of cardamom, allspice, chilli, cinnamon, clove, cumin, black pepper, nutmeg, sage, thyme, and their extracts on the growth of *Enterobacter aerogenes* and *Morganella morganii* in mackerel muscle extracts at 30°C. Clove and cinnamon showed the strongest antimicrobial effect against tested bacteria. Allspice, sage, and thyme powders and extracts were more effective against *M. morganii* than *E. aerogenes*. The antimicrobial activity of clove and cinnamon was apparently due to eugenol and cinnamaldehyde, respectively.

18.3.3 Antimicrobial effect of combined treatment of essential oils with other antimicrobial agents

The effica y of essential oils can be enhanced by their combination with other preservatives [28,47,65]. Mahmoud *et al*. [47,66] studied the preservative effects of a combined treatment with 0.5% carvacrol + 0.5% thymol (Cv+Ty) and electrolysed water solutions composed of cathodic solution (EW-) and anodic solution (EW+) on carp fillet at 5°C for 20 days. Combined treatment of the fillet with Cv+Ty and electrolysed solution resulted in a significan reduction ($P \leq 0.05$) in the total microbial counts immediately after treatment (day 0) as compared with the control. The total microbial count of control samples exceeded an acceptable limit (6.0 $\log_{10}$ cfu/g) after 4 days at 5°C. On the other hand, when the samples were pre-treated with electrolysed solution, and then treated with (Cv+Ty), the total microbial count exceeded the acceptable limit after 16 days at 5°C. Wendakoon and Sakaguchi [64] examined the combined effect of sodium chloride and clove on the growth of *Enterobacter aerogenes* in mackerel muscle. The presence of NaCl (2%) with clove (0.5%) completely inhibited the growth of *E. aerogenes* in mackerel broth, though clove alone was inactive on the growth of *E. aerogenes*. Later, Ouattara *et al*. [67] studied the combined effect of low-dose gamma irradiation and antimicrobial coating (thyme oil, 0.5 and 0.75%) and *trans*-cinnamaldehyde (0.3 and 1.5%) on the shelf-life of pre-cooked shrimp. A synergistic effect ($P \leq 0.05$) between gamma irradiation and coating treatments was observed. The shelf-life of treated shrimp was extended by 12 days compared to the control.

18.4 Conclusions

The most important compounds responsible for the antimicrobial activity of essential oils in fis or fis product were determined. Phenolic compounds were the main group responsible

for the antimicrobial activity of essential oils. Further investigations are needed to understand how phenolic compounds and other active compounds in essential oils interact with bacterial cells. In addition, interactions among different active compounds of essential oils and between these compounds and other nutrients of fis or fis products will be of continued interest to researchers working on the antimicrobial effect of essential oils. Hence, the needs to incorporate such challenging areas into the research and evaluate essential oils individually and in combination with other preservation techniques remains to be of priority. These may encourage the development of novel approaches in the preservation of fis and fis products.

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19 Rapid methods for the identification of seafood micro-organisms

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19.1 Introduction

Bacteria have an important role in the quality and safety of seafood. Spoilage considerations by select species dictate how fast fis and shellfis deterioration will occur. These bacteria compete with the flor under the parameters of temperature, atmosphere, and water activity (a_W). For researchers interested in bacterial load and diversity within the microbial flora more time is allotted for diluting and plating sample aliquots, isolating pure colonies, developing a culture collection, and conducting preliminary tests for determining which system and data base to use. Progress is being made towards rapidly determining the presence and quantity of food bacteria, specificall a range of pathogens. Researchers are taking cues from the food safety arena and are employing rapid molecular techniques to identify seafood spoilage bacteria and characterize the bacterial diversity of aquatic fis and shellfish The ability to rapidly identify bacteria can allow analysts to describe the changing microflor composition to predict shelf-life and diagnose potentially unsound products. This chapter includes aquaculture bacteria and is limited to non-pathogenic bacteria in seafood, since other chapters focus on the pathogenic bacteria (Chapters 16 and 23).

19.2 Non-molecular (phenotyping)

19.2.1 Analytab products (api®)

Test kits [1] identify bacteria within groups such as Gram-negative (GN) non-fermenters and Enterobacteriaceae or narrow them down to genera such as *Listeria*. Depending on the test kit employed, the diagnostic strips can range from 7 to 20 biochemical reactions. The kits are geared towards clinical microbiology laboratories, but have been found useful in certain studies involving seafood microbiology. For example, the gut microflor of hybrid tilapia was examined using these kits [2].

19.2.2 Biolog Inc.

Carbon utilization define some of the phenotypic characterization of micro-organisms and a commercially-available system [3] has streamlined the laboratory process. Simultaneous

use of 95 carbon-based substrates is tested and the results are subsequently compared to data bases for GN and Gram-positive (GP) bacteria using the MicroLog[TM] software. Over 1,000 species are contained in the bacteria data bases [4]. The microplates have found the greatest use in clinical microbiology and research laboratories devoted to microbial ecology. Seafood microbiology research using the GN and GP microplates has been limited to a handful of studies. Spoilage bacteria from four fis species were identifie using the GN microplates in this system [5]. The bacterial flor of aquacultured fis was identifie using GN and GP microplates [2,6–9]. Advanced phenotyping [10] using microarrays [4,11] has been carried out recently, but have not been used yet to identify seafood and aquaculture bacteria.

19.2.3 Microbial Identification Inc. (MIDI)

The structure of the cell wall as determined by fatty acid composition allows for speciation of aerobic and anaerobic bacteria using Sherlock® software [12]. Over 1,400 bacterial species are contained in the data bases [13]. Clinical microbiology laboratories have been the prime users of MIDI technology followed by environmental microbiology, while aquaculture and seafood microbiology research has employed MIDI sporadically. The fatty acid methyl ester (FAME) profile were recently used to differentiate four fis pathogenic bacterial species [14]. Another study evaluated MIDI for identifying spoilage bacteria during iced storage of Alaska pink salmon [15,16]. The bacterial flor of aquacultured fis was identifie using the FAME profile [6–9,17]. Analysis of 16S ribosomal (r) DNA [13,18] has enabled the company's efforts into genotyping.

19.2.4 Limitations for phenotypic identification of seafood and aquaculture bacteria

The above standardized identificatio systems have been designed primarily for the microbiology laboratories and the need for countless tests on patients in hospitals and clinics. Costs vary according to the approaches and software used, but the techniques are generally easy to accomplish (Table 19.1). Incubation times and temperatures are conducive for identifying bacteria in the sub-mesophilic range (28–30°C) to mesophilic range (32–37°C). Psychrophilic bacteria having optimum growth temperature below 20°C and being associated with cold-water aquaculture and seafood, cannot be identifie by the standard Biolog and MIDI protocols.

Table 19.1   Comparison of analytical methods for identifying bacteria

		Relative cost of instrumentation		
		High	**Medium**	**Low**
Technological sophistication	High	Biosensors, lab-on-a-chip, MALDI-TOF, microarrays, and ribotyping	DGGE, TTGE, MicroSeq, qPCR	None
	Low	Biolog, MIDI	API kits, PCR	Classical taxonomy

Note: for complete names of the method abbreviations, refer to the text.

19.3   Molecular (genotyping)

19.3.1   Polymerase chain reaction (PCR) and real-time or quantitative PCR (qPCR)

The PCR is a mature laboratory technique and now forms the basis of various molecular techniques described below. The advantage of qPCR over standard PCR is that no agarose gel electrophoresis followed by staining of separated bands is required. Amplifie genetic sequences accumulate and through the use of specifi dyes can allow the analyst real-time projections for cell quantification The molecular techniques vary in cost, depending on the level of sophistication used and equipment requirements (Table 19.1). Taxonomic analysis of aquaculture and seafood bacteria has taken advantage of these newer techniques and the number of publications reflect exponential growth (Fig. 19.1). Although the purposes differ for bacterial identificatio between aquaculture bacteriologists and seafood bacteriologists, the rapid molecular techniques are interconvertible. The former are concerned about microbial ecology and health or pathology of live fis and shellfis at different life stages, while the latter are occupied with spoilage and microbial diversity of harvested and processed seafood (Tables 19.2 and 19.3).

19.3.2   Molecular subtyping techniques

Denaturing gradient gel electrophoresis (DGGE), temporal temperature gel electrophoresis (TTGE), and single-strand conformation polymorphism (SSCP) are the latest innovations for separating species based usually on the 16S rDNA (gene) or sequencing the 16S rRNA molecule. Amplifie rDNA restriction analysis (ARDRA), intergenic spacer region (ISR) analysis, random amplifie polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and terminal (T)-RFLP are additional techniques for characterizing microbial communities. Details regarding these techniques, as applied to food bacteriology, have been reviewed [77–80] and will not be discussed further in this chapter. Aquaculture bacteria are usually identifie from 16S rDNA (Table 19.2), while seafood product quality
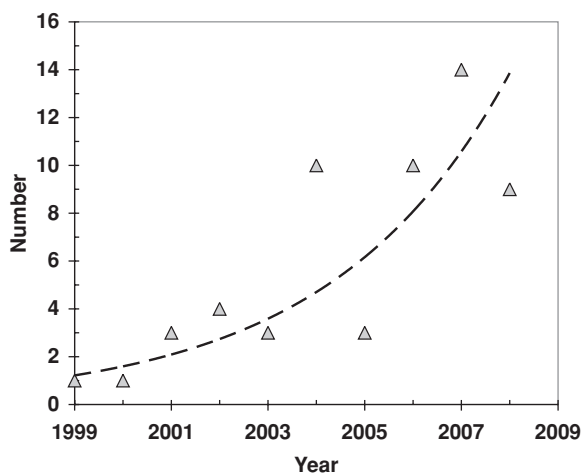
Fig. 19.1   Advancement of molecular biology techniques used for aquaculture and seafood bacteria identification represented by 58 journal publications (which are cited in Tables 19.2 and 19.3).

Table 19.2 Rapid molecular methods used for bacterial identification in aquaculture

Purpose	Bacteria identified	Isolated from	Methods	Reference
Larvae microflora	α- and γ-Proteobacteria	Cod	16S rDNA DGGE	[19]
Larvae probiotics	α- and γ-Proteobacteria	Turbot	16S rDNA RAPD	[20,21]
Larvae microflora	γ-Proteobacteria	Halibut	16S rDNA DGGE	[22]
Larvae microflora	γ-Proteobacteria	Halibut	16S rDNA RFLP	[23]
Gut microflora	γ-Proteobacteria	Halibut larvae	16S rDNA RFLP	[24]
Egg microflora	γ-Proteobacteria	Threadfin & amberjack	16S rDNA RFLP	[25]
Egg, larvae, and live feed microflora	Various	Cod & live feed	16S rDNA DGGE	[26]
Gut microflora	Various	Abalone	16S rDNA	[27]
Bacterial diversity	Various	Oyster	16S rDNA RFLP	[28]
Hatchery microflora	Various	Scallop larvae	16S rDNA DGGE	[29]
Intestinal microflora	γ-Proteobacteria & Actinobacteria	Salmon	16S rDNA ISR, TTGE	[30]
Intestinal microflora	Primarily γ-Proteobacteria	Salmon	16S rDNA RFLP DGGE	[31]
Intestinal microflora	Various	Salmon	16S rDNA DGGE	[32]
Intestinal microflora	Various	Salmon	16S rRNA	[33]
intestinal microflora	Various	Salmon	16S rRNA	[34]
Intestinal microflora	Various	Salmon	16S rDNA	[35]
Intestinal microflora	Various	Trout	16S rDNA DGGE	[36]
Intestinal microflora	Various	Trout	16S rRNA RFLP	[37]
Intestinal microflora	Various	Trout	16S rDNA RAPD	[38,39]
Intestinal microflora	Various	Cod	16S rRNA	[40]
Intestinal microflora	Various	Various fish	16S rDNA ARDRA	[41]
Intestinal microflora	Various	Bluegill	16S rDNA TGGE	[42]
Intestinal microflora	Various LAB	Salmon	16S rRNA	[43]
Intestinal microflora	Various LAB	Freshwater fish	16S rRNA RAPD	[44]
Intestinal microflora	Various Carnobacterium spp.	Cod	RAPD	[45]
Fish pathogens	Various LAB	Various fish & farms	16S rDNA ARDRA	[46]
Fish pathogen	Edwardsiella tarda	Fish culture system	16S rDNA RFLP	[47]
Fish & shellfish pathogens & probiotics	Various	Marine hatchery	16S rDNA DGGE	[48]
Finfish mortality	Primarily γ-Proteobacteria	Cod larvae	16S rDNA	[49]
Finfish mortality	Various	Haddock larvae	16S rDNA DGGE	[50]
Shellfish mortality	Various	Scallop larvae	16S rDNA ARDRA	[51]

Note: for complete names of the method abbreviations, refer to the text. LAB, lactic acid bacteria.

investigations target 16S rRNA sequencing or ribotyping (Table 19.3). In a novel study to track fis from aquaculture farms, the authors based the traceability on the bacterial 16S rDNA followed by DGGE [76]. An extension of DGGE has employed fluorescen probes to enable culture-independent bacterial flor analysis of meat and cheese [81]. In the future, a laboratory analyst may test a sample of fis or shellfish bypassing the essential bacteria

Table 19.3 Rapid molecular methods used for identification of spoilage bacteria and microflora diversity in raw seafood and products

Purpose	Bacteria identified	Isolated from	Methods	Reference
Spoilage bacteria	Pseudomonas spp.	Cod	qPCR	[52]
Spoilage bacteria	Pseudomonas, Shewanella, and Photobacterium spp.	MAP cod	16S rDNA DGGE	[53]
Spoilage bacteria	Pseudomonas, Shewanella, and Photobacterium spp.	MAP cod	16S rDNA DGGE	[54]
Spoilage bacteria	Pseudomonas, Photobacterium, and Brochothrix spp.	MAP halibut	16S rDNA DGGE	[55]
Spoilage bacteria	Pseudomonas fragi	Freshwater fish	16S rRNA sequencing	[56]
Spoilage bacteria	Shewanella spp.	Gilthead sea bream	16S rRNA sequencing	[57]
Spoilage bacteria	Shewanella spp.	Marine fish	16S rRNA sequencing	[58]
Spoilage bacteria	Lactobacillus and Photobacterium spp.	Cold-smoked salmon	16S rRNA sequencing	[59]
Spoilage bacteria	Various (some LAB)	Cold-smoked salmon	16S rRNA ARDRA & sequencing	[60]
Spoilage bacteria	Various LAB	Cold-smoked trout	Ribotyping	[61]
Spoilage bacteria	Various LAB	Gravad trout	Ribotyping	[62]
Spoilage bacteria	Leuconostoc spp.	Acidified herring	Ribotyping	[63]
Spoilage bacteria	Lactobacillus alimentarius	Marinated herring	Ribotyping	[64]
Spoilage bacteria	Lactobacillus spp.	Salted fermented herring	Ribotyping	[65]
Spoilage bacteria	Various (some LAB)	Charcoal-broiled lamprey	Ribotyping & 16S rRNA sequencing	[66]
Spoilage bacteria	Carnobacterium and Enterococcus spp.	Cooked & brined shrimp	16S rRNA sequencing	[67]
Microflora diversity	Various LAB	Various seafood products	16S rDNA ARDRA & sequencing	[68]
Microflora diversity	Various	Cod	16S rRNA T-RFLP & sequencing	[69]
Microflora diversity	Various	Cooked peeled shrimp	16S rRNA ISR & TTGE	[70]
Microflora diversity	Various	Cold-smoked salmon	16S rRNA ISR, RFLP & TTGE	[71]
Microflora diversity	Various	MAP salmon & coalfish	16S rRNA sequencing	[72]
Microflora diversity	Various	Puffer fish	16S rRNA	[73]
Microflora diversity	Various	Whiting	16S rDNA RFLP	[74]
Microflora diversity	Various	Zebraperch	16S rDNA RFLP	[75]
Traceability	Not described	Aquacultured fish	16S rDNA DGGE	[76]

Note: For complete names of the method abbreviations, refer to the text. LAB, lactic acid bacteria and MAP, modified-atmosphere-packaged fish products.

cultivation and purificatio steps, and have the identificatio authenticated to genus, species, and biotype within hours.

19.3.3   Commercially-available systems

Ribotyping or analysis of 16S rRNA is conducted using the RiboPrinter$^{®}$ [82]. This and the MicroSeq$^{®}$ Microbial Identificatio system [83] have found use in government, academic, and commercial analytical laboratories. The MassARRAY$^{®}$ with iSEQ^{TM} software system has been developed [84] and employs matrix-assisted laser desorption-ionization time-of-fligh (MALDI-TOF) mass spectrometry [85]. The DiversiLab^{TM} System [86–88] and microarrays are becoming more familiar regarding identificatio of bacteria. We are unaware if these systems, with the exception of ribotyping (Table 19.3), have been tested on seafood spoilage bacteria and identificatio of aquaculture bacteria.

19.3.4   Polyphasic taxonomy

The principles of polyphasic taxonomy [89,90], combining phenotyping and genotyping, strengthen the identification validation, and bacteria phylogeny over any single approach. The American Type Culture Collection [91] employs many of the above techniques when verifying new bacterial species submitted to the organization and before these clones are officiall accepted for deposit. For example, classical taxonomic tests, API 20E kits (for non-fermenting GN bacteria), and sequencing of the 16S rRNA gene were used to identify potentially pathogenic species of *Shewanella* and *Photobacterium* isolated from oysters and seawater [92]. Similarly, classical taxonomic tests, GN microplates, and sequencing of the 16S rRNA gene were used to identify a new pathogenic bacterium of aquacultured gilthead sea bream [93].

19.4   Conclusions

Each of the rapid techniques has specifi advantages and disadvantages for the laboratory analyst to decide on when selecting a system or systems for identifying seafood micro-organisms. Time, cost, and advanced technical training are the major factors that have controlled identificatio techniques. Phenotypic characterization through metabolic reactions, carbon utilization, and FAME analysis supplanted the classical taxonomic tests. Interest has emerged, from the work in clinical and biosafety laboratories, for understanding the diversity of the seafood microbial flora particularly the pathogenic bacteria through the use of molecular techniques. Aquaculture facilities and commercial seafood industries may contact analytical laboratories that use the above techniques to identity problematic bacteria in seafood farms, processing plants, and products or for confirmin a healthy micro-ecosystem exists. Advances in forensic bacteriology and whole genome sequencing analysis may become tools for furthering the rapid identificatio of seafood and aquaculture bacteria that are culturable or culture-independent.

19.5   Acknowledgements

are not endorsements by the authors, but are shown as examples of potential uses for identifying seafood spoilage and aquaculture bacteria.

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20 Using predictive models for the shelf-life and safety of seafood

Graham C. Fletcher

20.1 Introduction

In terms of quality, the seafood industry needs to know what their products will look, smell, and taste like when it is consumed next week in the case of a chilled product, or next year in the case of a frozen product. With regard to microbial safety, the seafood industry needs to not only know that its products are safe now but will be safe next week when their customers eat them. However, because tests take time to complete, microbiologists can only confir that a product was safe a few hours, or more often, a few days ago. For the seafood industry, predicting the future is not an optional feature. It is an essential for doing business. It is this need that predictive modelling of seafood shelf-life and safety is designed to address.

Although great progress has been made in the science and mathematics of predictive food modelling over the last four decades, it is beyond the scope of this chapter to review all of this. Books have been written on this subject [1–3] and international conferences [4] provide effective reviews of advances in the area. This chapter highlights predictive models that have been developed specificall for seafood.

Two main types of models are usually used in predictive modelling.

1) Primary models indicate the effect of time on a particular attribute (e.g. bacterial number) under particular constant processing or storage conditions (e.g. 0°C storage).
2) Secondary models indicate how parameters derived from the primary model (e.g. the bacterial growth rate) change in response to the product being held under different constant processing or storage conditions (e.g. temperatures between –5 and 20°C).

A number of predictive models for food have been incorporated into software packages (so-called tertiary models). The user can input their product and environmental parameters and obtain predictions of microbial numbers or product quality at selected times. Dalgaard [5,6] has produced and made freely available an invaluable piece of software for predicting seafood safety and spoilage under various conditions (Fig. 20.1). The software contains 15 models to predict the shelf-life and safety of seafood and can be accessed in 15 languages. The Fish Shelf-life Prediction Program [7], an add-in for Excel, is also freely available. It models

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spoilage bacteria and sensory acceptability of sea bream and turbot [8]. Generic software that is not specifi to seafood is also available for predicting the growth of microbes under various conditions, including the freely available Pathogen Modelling Program (PMP) [9] and ComBase (Combined data base on predictive microbiology information) [10] packages. Ten other predictive microbiology software packages are briefl reviewed in the help pages of Seafood Spoilage and Safety Predictor (SSSP) [5]. Many of these generic models have been generated in laboratory media and the results should be verifie for application to particular seafood.

20.2   Predicting contamination

The firs step in quantifying seafood safety issues is often to quantify the initial level of contamination. As models to predict this are difficul to develop, most predictive models require the user to estimate the initial level of contamination based on product sampling. However, a few attempts have been made to predict initial contamination levels. An initial model by the US Food and Drug Administration (FDA) for the levels of *Vibrio parahaemolyticus* based on water temperature was incorporated into their risk assessment for raw oysters [11]. Subsequently, a more extensive model incorporating chlorophyll levels and turbidity was developed. These parameters explained some of the variations from predictions using the earlier model [12]. Gardini *et al*. [13] modelled the effect of environmental parameters (season and geographical origin) on the levels of contamination by fecal coliforms or *Escherichia coli* in baby clams (*Chamelea gallina* L.). This allowed them to predict the frequencies of samples with concentrations of the indicator organisms below or above the legal standards. Later, Aarnisalo *et al*. [14] modelled the transfer of *Listeria monocytogenes* to salmon during slicing with a contaminated slicing blade. The quantity of *L. monocytogenes* decreased exponentially with subsequent slices and this was dependent on the contamination level and the temperature. Despite these attempts, initial levels of contamination generally remain difficul to predict from mathematical models.

20.3   Predicting microbiological safety in chilled storage

20.3.1   Histamine production

Histamine or scombroid poisoning occurs when bacteria convert naturally occurring histidine into histamine. One of the firs predictive models for seafood safety was that of Frank *et al*. [15], who, before personal computers were widely available, published an effective graphical method for estimating histamine formation in skipjack tuna held at elevated temperatures. Much later, Emborg and Dalgaard [16,17] modelled the effect of a range of parameters (temperature, CO_2, a_w, and pH) on growth and histamine production of psychrotrophic *Morganella psychrotolerans* and mesophilic *Morganella morganii*. For validation, their model was evaluated by applying it to the results of various studies on histamine production in fis described in the literature. Based on this work, Dalgaard [5] subsequently included two models in the SSSP software. The firs (Fig. 20.1) predicts growth and histamine production of both *M. morganii* and *M. psychrotolerans* based on storage temperature. The second is based solely on the response of *M. psychrotolerans* to refrigerated storage temperatures, gas atmospheres, salt levels, and/or pH.
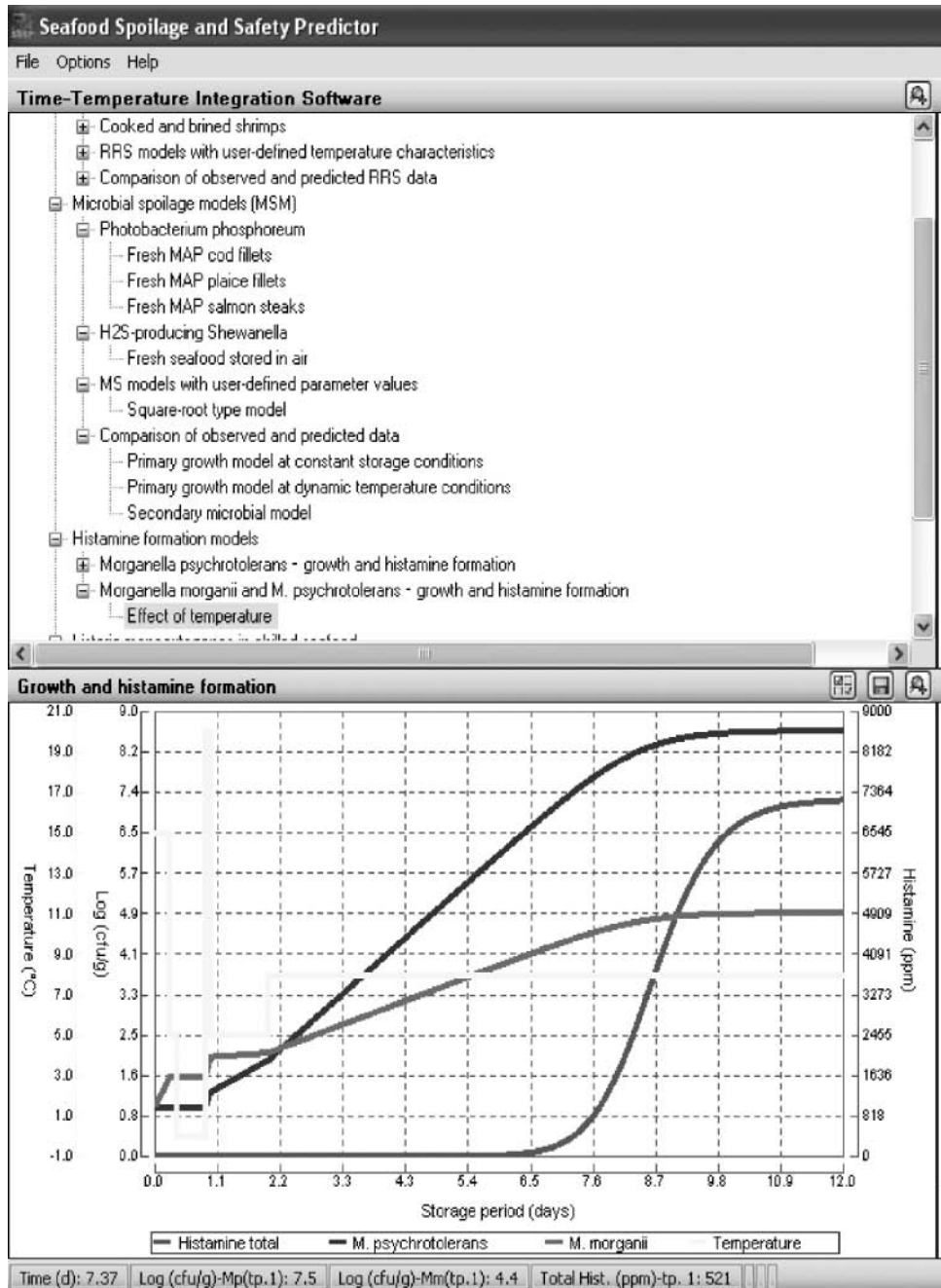
Fig. 20.1 Seafood spoilage and safety predictor: part of the output when modelling histamine production using varying temperatures. Adapted from Dalgaard *et al.* [6].

20.3.2 Growth of *Listeria monocytogenes* in lightly preserved seafood

By far, the most published seafood-related predictive modelling work has been on the growth of the food-borne pathogen *L. monocytogenes* and most of this in a single product, namely cold smoked salmon (CSS). Initially, Dalgaard [18] proposed an iterative approach to modelling *L. monocytogenes* in CSS, combining different kinetic and empirical models. Next, Thurette *et al*. [19] developed a model to predict the behaviour of *Listeria* as a function of temperature, salt, and amount of liquid smoke expressed as phenol concentration. They validated their model with challenge tests on smoked fis and found agreement between predicted and experimental values within 0.5 log for 60% of the results. Then, Gimenez and Dalgaard [20] developed a model based on the cooked meat model of Devlieghere *et al*. [21], but incorporating the effect of smoke components. Afchain *et al.* [22] developed a statistical model of the effect of dynamic temperature on growth of *L. monocytogenes* in CSS that took account of a_w and pH. About the same time, Augustin *et al.* [23] evaluated models for their ability to describe the behaviour of *L. monocytogenes* in seafood products. They concluded that models excluding interactions were sufficien to predict growth rates, while interactions had to be included to predict growth–no-growth boundaries. They proposed a new model to predict confidenc limits for the growth rate of *L. monocytogenes*. Cornu *et al.* [24] evaluated the effect of temperature, water-phase salt, and phenolic contents on the growth of *L. monocytogenes* in CSS. They concluded that the secondary model proposed by Devlieghere *et al.* [21] and modifie by Gimenez and Dalgaard [20] was appropriate but also noted that further research was needed to understand all of the factors affecting growth of *L. monocytogenes* in CSS and to obtain fully validated predictive models for use in quantitative risk assessment. Subsequently, Delignette-Muller *et al.* [25] developed a Bayesian model to predict the simultaneous growth of *L. monocytogenes* and food flor in CSS with a competitive model expressing variability and uncertainty. Next, Mejlholm and Dalgaard [26] modelled the effect of diacetate and lactate against *L. monocytogenes* in vacuum packaged or modifie atmosphere packaged (MAP) CSS, marinated salmon, cold-smoked Greenland halibut, marinated Greenland halibut, and gravad salmon. About the same time, Beaufort *et al*. [27] developed a model that also included the effects of microbial competition on growth of *L. monocytogenes*. After this, Hwang [28] evaluated the effects of salt, smoke compounds, storage temperature, and their interactions on the growth of *L. monocytogenes* in CSS, developing two models to describe the lag phase duration and growth rate in salmon containing 0 to 8% salt and 0 to 34 ppm of phenol at storage temperatures of 4 to 25°C. About the same time, Pouillot *et al*. [29] developed a model to predict exposure of consumers of French CSS to *L. monocytogenes*, which takes into account bacterial growth and interactions with other microflor Their model suggests that the most important factor affecting consumer exposure is the prevalence of the organism in the product and the household refrigerator temperature. More recently, Chitlapilly *et al.* [30] claimed that for vacuum-packed Irish CSS, the Arrhenius type model was able to interpret the effect of temperature better than the square root model. Their experimental data were in line with the predictive software models in ComBase Predictor and the Pathogen modelling programme at 4 and 10 °C [30].

The growth–no-growth boundary is a special condition of microbial growth models that deserves separate treatment. In their SSSP software, Dalgaard [5] and his team incorporated a growth–no-growth model predicting this boundary for *L. monocytogenes* under seven environmental parameters (temperature, atmosphere (CO_2), water phase salt/a_w, pH, smoke

components/phenol, lactate, and diactate) in lightly preserved seafood. A mathematical model was developed that included the effect of microbial growth inhibitors (diacetate, lactate, CO_2, smoke components, nitrite, pH, NaCl, and temperature) and interactions between all these inhibitors to predict the growth boundary of *L. monocytogenes* in lightly preserved seafood. They went on to model the effect of these growth inhibitors on the growth of lactic acid bacteria (LAB) in the lightly preserved seafoods and improved their *L. monocytogenes* model by taking into account the effect of microbial interaction with LAB [31]. This model was further developed into a very complex 12 parameter generic model for growth–no-growth of *L. monocytogenes* in lightly preserved seafood, which has been incorporated into the SSSP software. The model was validated and shown to perform better than other models, firs for ready-to-eat shrimp [32] and then most recently in over 600 studies on lightly preserved seafood, meat, poultry, and dairy products [33]. The model found in the SSSP includes values of ψ, an estimate of how close a particular formulation is to the growth–no-growth boundary and, therefore, a measure of confidenc in predictions of growth–no-growth. Based on the validation studies, Mejholm *et al.* [33] recommend that products are developed with a ψ value of at least two.

20.3.3   Toxin production by *Clostridium botulinum*

Another safety issue that has received attention from predictive modellers is the issue of the growth and toxin production of psychrotrophic *Clostridium botulinum*, particularly Type E. Along with *L. monocytogenes*, this is one of the few food poisoning organisms that can grow at refrigeration temperatures. The heat resistant spores of *C. botulinum* Type E naturally occur in many seafoods, but as an anaerobic species it is usually only of concern in cans, vacuum packs, or MAP with reduced O_2. Early on, Genigeorgis [34] identifie that *C. botulinum* would be a major issue in MAP and with his co-workers [35–40] he went on to develop probability models for the growth and toxin production of this organism in a range of products including seafood. Based on this work, Skinner and Larkin [41] developed a conservative relationship to predict the lag time for botulinum toxin production with the intention that this be used in conjunction with a time-temperature indicator (TTI). Later, Mendoza *et al.* [42] evaluated three commercially available and fi e prototype TTIs. Performance was monitored to determine the Arrhenius parameters of the TTIs and models were used to predict their performance under dynamic thermal conditions. They concluded that three of the TTIs could be used to predict the botulinum safety of fresh seafood in reduced oxygen packaging.

20.3.4   Other hazards

Few modelling studies have been done on seafood hazards other than for histamine production in scombroid fish *L. monocytogenes* in lightly preserved seafood, and *C. botulinum* in packaged seafood. Giuffrida *et al.* [43] attempted to model the growth of pathogenic *Aeromonas hydrophila* on fis surfaces during storage. Their model simultaneously took into account the environmental fluctuation and the bacterial interspecifi competition based on mesophilic aerobic plate counts. Working in broth and oyster slurry, Yoon *et al.* [44] developed primary and secondary models for lag time and growth of both pathogenic and non-pathogenic *V. parahaemolyticus* in response to temperature and time. The oyster slurry gave less growth than laboratory broth, and experimental observations gave similar growth rates and longer lag times than the slurry model predicted. Zhou *et al.* [45] modelled the

effects of temperature, a_w, and pH on growth of *Streptococcus iniae* in tilapia. Predictions were satisfactory and they suggested that their model could be used for risk assessment.

20.4 Predicting spoilage and shelf-life in chilled storage

20.4.1 The square root model as a secondary model

Seafood researchers have played a leading role in the development of food models to predict shelf-life. In 1971, Nixon [46] was one of the firs to describe a relative spoilage rate function that would enable the effects of different temperatures on spoilage to be compared. Olley and Ratkowski [47,48] refine Nixon's preliminary estimate of this function to one based on the Arrhenius kinetics developed from 70 literature data sets pertaining to fis spoilage. Ratkowski and Olley [49] later realised that their data set was better explained by a square root or Bĕlehrádek model and this model has subsequently been implemented into the relative rate predictive software on fis spoilage [5]. The square root model not only gave seafood researchers the basis for integrating the effect of time and temperature on fis spoilage and to enable the prediction of remaining shelf-life, but it also found applications in a wide range of situations and stimulated the development of increasingly comprehensive predictive microbiology models [3,50,51].

20.4.2 Linear responses as primary models

In modelling spoilage, the concentrations of a few chemical compounds (e.g. hypoxanthine, see Chapter 3) increase linearly with chilled storage. Other indices [52] based on the whole pathway of adenosine 5′-triphosphate (ATP) catabolysis to hypoxanthine (e.g. the K-value) also give a linear response with time (Chapter 2). These indices can then be used to predict time to end of shelf-life.

Another example of a linear response with storage is that of the quality index method (QIM) for inspecting whole fis [53]. This provides a more powerful tool than traditional fish-gradin techniques. Once a particular QIM score is established as the limit of commercial shelf-life, the time to reach this limit can be predicted with a degree of certainty for fis stored in ice. Times in ice can then be converted to times at any other combination of temperatures using the square root model, which is available in the SSSP software [5].

20.4.3 Specific spoilage organisms

Gram and Dalgaard [54] has pursued seafood spoilage models based on the concept of specifi spoilage organisms (SSO). The assumption is that under a given set of circumstances one micro-organism will be the dominant spoiler, so modelling this organism can allow spoilage to be predicted. In his PhD thesis, Dalgaard [55] developed a predictive models for aerobically stored seafood based on sulphide-producing *Shewanella* species. The models were validated with respect to cod, haddock, hoki, orange roughy, smooth oreo dory, sea bream, and snapper [56]. Dalgaard's view [56] was that both empirical and microbial spoilage models might predict shelf-life accurately but only in specifi seafoods stored within define conditions.

20.4.4   Microbial growth under modified atmosphere packaging

A number of the spoilage models for seafood include seafood under MAP. Dalgaard *et al*. [56–59] developed models for three fis products (cod fillets plaice fillets and salmon steaks) using *Photobacterium phosphoreum* as the SSO. These models have been included in the SSSP software [5], but do not include the effect of using O_2 in the gas mixes, which is recommended for some of these species [60].

A group of Greek researchers has explored the modelling of spoilage of Mediterranean fis species. Using Mediterranean fis red mullet (*Mullus barbatus*) as a case study, they developed models combining the effect of temperature with the level of CO_2 in a modifie packaging environment [61]. The growth of a range of spoilage flor (*Pseudomonas* spp., *Shewanella putrefaciens*, *Brochothrix thermosphacta*, and LAB) was monitored and combined models developed. The models were assessed on three different fis species, and the Arrhenius and Belehradek models were judged satisfactory. Having established *Pseudomonas* spp. as the SSO of aerobically stored Mediterranean gilt-head sea bream (*Sparus aurata*) [62], Koutsoumanis [63] developed a model to predict growth rate and lag phase for this group of organisms at different isothermal temperatures using the Belehradek (square root) relationship. He validated the model under dynamic non-isothermal conditions. Working on marine-cultured sea bass (*Dichentrachus labrax*), the research group found sensory shelf-life was correlated to *Pseudomonas* numbers of 10^7 CFU/g [64]. Kinetic models, shelf-life correlations, and N^0 count at time 0 were incorporated into a shelf-life decision system for sea bass. Subsequently, Simpson *et al*. [65] developed a model to predict the consequences of temperature abuse on the shelf-life of Pacifi hake in MAP. The model predicts the effects of temperature, gas concentration, and relative humidity on shelf-life. Then Corbo *et al*. [66] developed a kinetic model based on total bacterial count and total coliforms to evaluate the shelf-life of cod under different MAP treatments (high and low oxygen) and temperatures.

20.4.5   Use of time-temperature indicators

Greek researchers also applied their predictive models to assess the applicability and usefulness of TTIs as effective tools of chill chain monitoring. First they modelled the effect of temperature on both the growth of the SSOs (*Pseudomonas* spp. and *S. putrefaciens*) in boque (*Boops boops*) and the response of several TTIs [67]. Then, when evaluating TTIs as a reliable management tool that matched predictive spoilage models for gilt-head sea bream (*Sparus aurata*), they conclude that practical difficultie and limitations still need to be addressed [68]. Next, Tsironi *et al*. [69] established a kinetic model for growth of spoilage bacteria (total flor and LAB) on vacuum packed tuna slices and evaluated the applicability of a TTI to improve quality. Sampling results validated their model and the management system incorporating the TTI gave improved outcomes. About the same time, Nuin *et al*. [8] evaluated the ability of a TTI to indicate the end of shelf-life when modelling spoilage of fresh turbot. Sensory, microbiological assessments and the TTI all correlated well. Their results were incorporated into the Fish Shelf-life Prediction Program [7].

20.4.6   Instrumental methods to detect spoilage

As well as modelling the growth of spoilage micro-organisms directly, a number of studies have begun to model the development of their spoilage products directly. Olafsdöttir *et al*. [70] evaluated the influenc of temperature on the microbial spoilage of haddock fillets and

developed a model to predict sensory quality using electronic nose sensors (CO, NH$_3$, and H$_2$S), *Pseudomonas* counts and a time-temperature variable with good agreement between the predicted and experimental data. Recently, Limbo *et al*. [71] investigated predictive methods for European sea bass based on storage temperature using both chemical (total volatile base-nitrogen (TVB-N) and thiobarbituric acid-reactive substances (TBARS)) and olfactometric (electronic nose) methods rather than microbiological tests. In another recent study, Tsironi *et al*. [72] developed a model of the effects of osmotic dehydration and temperature on quality indices (colour, microbial growth, TVB-N, and sensory scores) for gilt-head sea bream fillets

20.5   Predicting spoilage and shelf-life in frozen storage

The situation with frozen stored seafood is a little less complicated than with chilled product. When product is stored below –10°C, micro-organisms do not grow and safety is not compromised. The only issue is spoilage through chemical processes (oxidation and autolytic enzymes), which occurs with increasing slowness as the temperature drops. These chemical processes are usually taken to occur as first-orde reactions that can be described by the Arrhenius relationship. For example, Dyer and Hiltz [73] investigated the degradation of ATP derivatives through to hypoxanthine in frozen sword fish and found that the Arrhenius plot was linear for temperatures between 0 and –26°C. They concluded that the hypoxanthine content of the white muscle could be used to predict quality using the Arrhenius relationship. Recently, Tsironi *et al*. [74] modelled the shelf-life of frozen shrimp under variable conditions. They also found that the Arrhenius equation adequately modelled shelf-life based on changes in colour, TVB-N, trimethylamine (TMA), and sensory characteristics with activation energies between 118 and 156 kJ/mol. The model was validated under fluctuatin time-temperature conditions.

20.6   Predicting inactivation

Thermal inactivation was firs modelled in the food industry to protect consumers from the hazards associated with *C. botulinum* Type A in heat-processed products such as shelf stable cans. Log-linear primary and secondary models (the Bigelow model [75]) were used and conditions applied where the model predicted at least 10^{12} spores would be killed. The use of this predictive model has served the canning industry well and no incidents of botulism have occurred as a result of toxin production from spores surviving these treatments. Similar models have been applied to inactivating *Listeria* (the most heat-resistant vegetative pathogen of concern) in seafood with recommendations for 6 to 7 log$_{10}$ reductions in counts [76]. Inactivation must be modelled in each seafood product as inactivation of micro-organisms is affected by the surrounding medium [77,78]. "D" and "z" values for *L. monocytogenes* have been calculated for blue crab [79], cod [80], salmon [80], fresh mussels [81], and mussels prepared for hot smoking [82]. Other work has investigated the thermal inactivation of histamine-producing bacteria [78,83], but because *Listeria* is more heat resistant to thermal inactivation and may also be present in products that cause histamine poisoning, heat processes predicted to eliminate *L. monocytogenes* are recommended for heat processing seafood [76,84]. The Bigelow model has also been applied to determine the effectiveness of marinades [77] and high pressure processing
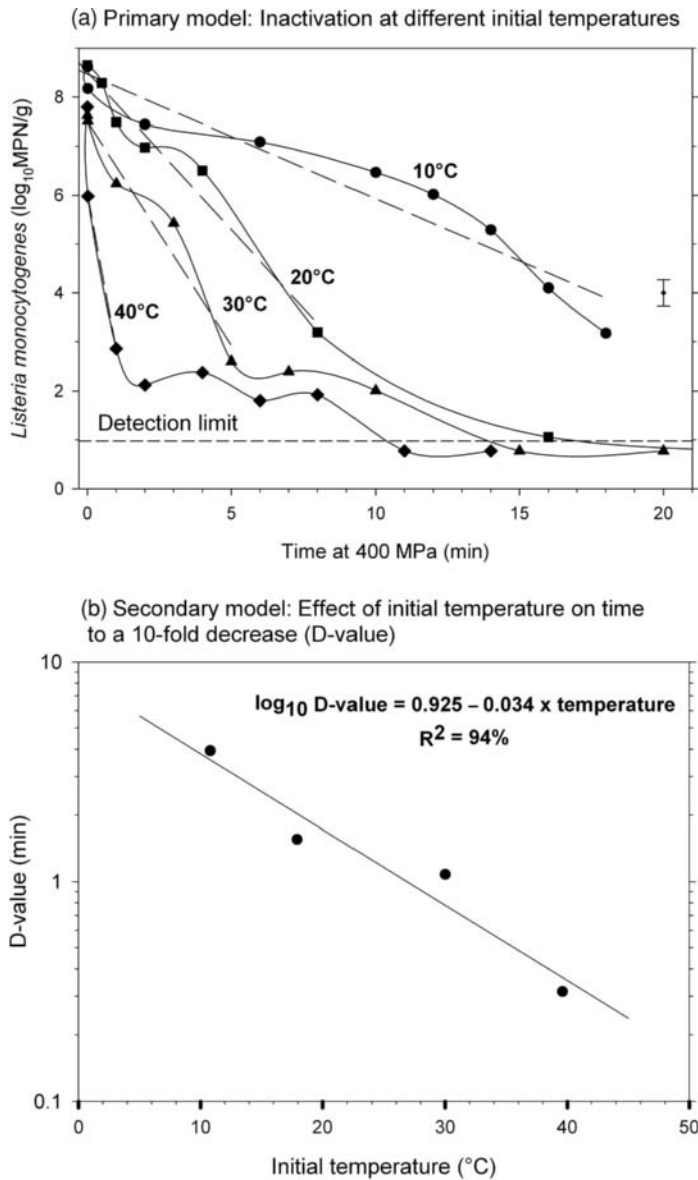
Fig. 20.2   Models for the effect of initial temperature on the inactivation of *Listeria monocytogenes* by high pressure processing at 400 MPa. Adapted from Fletcher *et al.* [85], with permission of Taylor & Francis.

(HPP) at 400 MPa [85] to inactivate *L. monocytogenes* (Fig 20.2). Although microbial inactivation is often not log linear, all the available models for thermal inactivation of bacteria in seafood use log linear models of inactivation.

The data presented in Fig. 20.2 also highlights some of the limitations of the predictive models. The model was developed on *Listeria* in minced mussel meat but *Listeria* on the surface of a mussel may behave differently. Although 5 $\log_{10}$ reductions in *Listeria* were achieved in a log linear fashion, beyond 5 logs, the inactivation data shows a tailing

phenomenon. Thus, if a 7 $\log_{10}$ reduction was required, the relationship presented in Fig. 20.2b would not apply, but a model that accounted for the tail would have to be developed. Also, the data in Fig. 20.2a (particularly at 10°C) suggest that there may be some convex curvature to the inactivation, so the model based on linear inactivation may be inaccurate. Thus, although the secondary model predicts times for inactivation of *Listeria* using HPP, careful consideration of the limitations of the model is needed. For critical processes, the model must be validated with challenge tests using the actual products and processing conditions to which it is to be applied. The same applies to virtually all models available for predicting the safety and shelf-life of seafood. Although they give predictions, the accuracy of those predictions must be tested.

20.7   Conclusions

Thirty years ago, apart from models on thermal inactivation of bacteria for safety in canning, predictive modelling of foods did not exist as an area of science. Today, the seafood industry can use a range of models to answer questions about the safety and shelf-life of their products. Most predictive models focus on bacteria, but more research is needed on non-bacterial aspects of seafood safety and spoilage, including autolytic enzymes, oxidative processes, sensory properties, chemical toxins, and spoilage products, etc. Some of the available models can be accessed as software packages while others must be applied directly from mathematical formulae. Using basic information supplied by the seafood producer about the characteristics of their product, in many cases the models can predict the status of the product after a preservation process has been applied, or after it has been stored for one week or one month under known conditions. However, the accuracy and effectiveness of such predictions have seldom been well tested and many of the predictive models have been developed on a limited number of parameters for a single seafood product. Their applicability in a wider range of products or after changes in product formulation has yet to be tested. Those using such models to predict seafood safety and shelf-life should apply these models judiciously. Users need to be careful to read and understand the research on which the models are based and consider the limitations of that research. They should also carry out their own studies validating model use in their own particular circumstances. The exception to this might be the predictive model on *L. monocytogenes* in chilled seafood available in the SSSP software package [5]. This model has been extensively tested in an international validation study and indications are that it can be used in a fail safe manner in a wide range of circumstances. However, the publishers of this model still accept no liability for the consequences of its use [5]. Thus, models are available and can usefully be used for initial predictions of seafood shelf-life and safety. More complex models, which include more parameters and have been subjected to more international validation studies, are generally needed before predictive models can become the sole basis for making fina decisions in critical areas such as food safety.

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21 Mathematical modelling of shrimp cooking

Ferruh Erdoğdu and Murat Ö. Balaban

21.1 Introduction

Observation (based on the availability of experimental data) or physics-based mathematical models play an important role in describing process simulations. As reported by Datta [1], mathematical modelling of food processing operations has been widely used, starting with Texieira *et al*. [2]. Physics-based mathematical modelling is based on fundamental mechanisms of physics governing the process and solving the differential equations describing that process [3]. By using mathematical models, experimental methodologies can be carried out virtually with the advantage of obtaining results for different conditions in an easier, quicker, and economical way.

Study of heat transfer in food processing is significant since thermal processing is still the most common method employed in preservation and consumption purposes of food products. It is mostly applied for cooking, pasteurisation, and sterilisation, where each process has its specifi objectives provoking physical and chemical changes in the product [4]. Mendez and Abuin [4] reviewed the thermal effects on the quality changes of seafood products during the process. Thermal processes reduce the number of micro-organisms in the product, change quality attributes affecting sensory, textural, thermal, and physical properties (moisture content, density, thermal conductivity, volume change, and shrinkage, etc.). They also cause cook losses, an important economic consideration, due to changes in moisture content as a result of modificatio in the structure of proteins [5]. During thermal processing, proteins denature and lose their water holding ability, and this is accompanied by dimensional changes and cook loss. Erdoğdu *et al*. [6] reported significan effects of internal temperature increase on cook losses in shrimp. Effect of temperature increase is accompanied by denaturation of myofibrilla proteins and shrinkage of collagen, leading to tightening and stiffening of the muscle [7]. Simultaneously, drip loss occurs with thermal effect, leaving voids among muscle fibre and results in the change of overall density and volume [8]. Murakami [9] reported significan moisture loss and shrinkage in the blanched shrimp and scallops. Erdoğdu *et al*. [10] determined the shrinkage starting temperature of shrimp around 70°C with cook loss ranging from 1 to 40% when cooking in water at 55 to 95°C with moisture loss and dimensional shrinkage values reaching up to 40% [11]. In fact, sarcomer units of the myofibrilla proteins were reported to start shrinking at a higher rate

when the temperature was over 70°C [12], leading to higher shrinkage and cook loss. Cook loss and its minimisation to a desirable level are significan economical considerations in the seafood industry [13]. Phosphates might be used to reduce moisture and cook loss by increasing water holding capacity of the proteins [14]. They promote the interaction between protein and water molecules due to increased pH and ionic strength; and increase in protein water interaction raises solubility and water holding capacity of proteins [14]. Even though phosphates are used to decrease cook loss, characterising the effects of thermal treatment and understanding dynamics of moisture loss leading to cook loss are important for process control and yield impovement of the fina product [15].

Erdoğdu *et al*. [10] suggested that cook loss can be predicted by knowing the temperature distribution of the product for process design and optimisation purposes. Using temperature history of the product, microbial lethality kinetics for a micro-organism present in the product (with known D- and z-values) can also be evaluated for safety consideration in addition to the cook loss. Reductions in microbial load and cook loss are two important criteria that can be used in establishing proper optimum cooking schedules [16]. Hence, prediction of temperature distribution inside the product becomes a significan modelling requirement for optimum cooking.

The easiest approach to apply in modelling purposes to determine temperature distribution is the use of exact (analytical) or numerical solutions for the required differential equations describing a given process. This chapter summarises the use of exact and numerical solutions for modelling purposes and presents the use of a numerical solution methodology to determine temperature change for optimum design of shrimp cooking.

21.2   Exact solutions

Exact solutions of partial differential equations are obtained using separation of variables, Green functions, and Laplace transform solution techniques. They play a significan role in heat transfer simulations for design and optimisation purposes where solid food products can be approximated by regular shapes of slab, cylinder, or sphere with constant thermal and physical properties [17]. The solutions are available in the literature, to obtain transient temperature distribution in such shaped foods. Carslaw and Jaeger [18] compiled a large number of analytical solutions for different situations for different shaped geometries. Separation of variables, based on expanding a function in Fourier series, has been the most widely used methodology. However, exact solutions, as indicated, are only available for regular shaped objects, and incorporation of dimensional changes into these solutions is difficult

The exact solutions for slab, cylinder, and sphere have also been reduced to relatively simple charts such as Heisler charts and Gurney-Lurie charts. These charts were the plots of dimensionless centre temperature ratio of the regular shaped geometries versus Fourier number as a function of the inverse of Biot number. Jaczynski and Park [19] used Gurney-Lurie transient temperature charts, derived from analytical solution of conduction heat transfer for an infinit slab, to simulate temperature changes during thermal processing of surimi seafood with known values of thermal diffusivity, thermal conductivity, and heat transfer coefficient

In most cases, thermal and physical properties of seafood products change with respect to temperature and moisture content of the product, in addition to the dimensional changes during the thermal process. These result in difficultie and complexities in deriving the exact solutions. Sometimes, based on the given circumstances, it might not be possible to derive

an exact solution. Therefore, using a numerical solution method would be a better choice to incorporate the changes in dimensions and thermal and physical properties.

21.3   Numerical solutions

In mathematical modelling studies, applying numerical solutions is a useful approach in solving the governing partial differential equations for the given process. They give additional advantages over exact solutions, since they can be applied over irregular geometries with variable thermal and physical properties during the process.

Numerical solutions make discrete mathematical approximations of the differential equations by spatial variations and time to transform them into a series of difference equations to be solved by different mathematical procedures. Since the solution requires a discrete number of points (so-called grid) as a consequence of discretisation, the result becomes an approximation rather than an exact solution [20]. The error in this approximation can be decreased by reducing the grid size (increasing the number of volume elements de-fragmenting the given object) at the expense of computation time [20]. For numerical solutions, two approaches are applied with their own advantages and disadvantages, depending upon the nature of the problem: finit difference and finit element methods. While finit difference methods have been widely used for regular shaped geometries, finit element methods are applied for the case of irregular ones. Mokhtar *et al.* [21] used an explicit finit difference numerical methodology to model the heat transfer problems of fis packages during precooling stage. Simpson *et al.* [22] also developed a mathematical model for mackerel packed in a retortable pouch using a finit difference method for process optimisation. Modelling with finit element methodology is more complex compared to finit difference methods, and sometimes using commercial software might be required. Zhang *et al.* [23], for example, used the finit element software *FIDAP* 8.52 (Fluent Inc., Lebanon, NH, USA) for numerical simulation of pre-cooking and cooling of skipjack tuna. Besides finit difference and finit element methods, computational flui dynamics (CFD) methodology is another powerful numerical tool that is becoming widely used for modelling purposes by relieving researchers from the difficult of code writing [1]. Computational procedure of CFD methodologies is based on using finit difference, finit element, and also finit volume methods. The latter one combines the fl xibility of the finit element method with the execution speed of the finit difference method [20]. Recently, Niamnuy *et al.* [24] modelled coupled transport phenomena and mechanical deformation of shrimp during drying using the *COMSOL Multiphysics*TM 3.3a (Comsol AB, Stockholm, Sweden) software.

Numerical methods are more efficient but might be more complex compared to the exact solutions. However, when properly applied, they might be easily used for further design and optimisation of cooking schedules of different foods including seafood products. Based on this, detailed information for developing a numerical model for shrimp cooking and further use of this model to determine product safety and cooking optimisation are presented below.

21.4   A numerical model for shrimp cooking

The firs step in developing a numerical model is to decide about the product geometry. In the case of shrimp, this becomes crucial and extra difficul since shrimp geometry does not
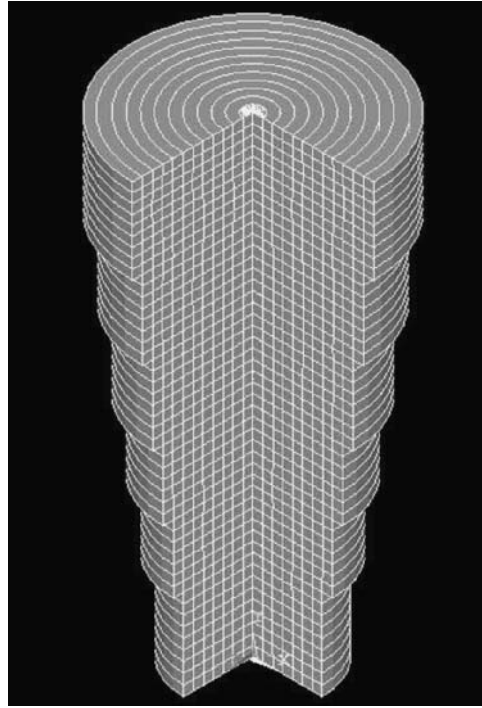
Fig. 21.1   Shrimp geometry approximation used in the numerical finite difference model.

conform to a regular geometry. For this purpose, Erdoğdu *et al.* [10] measured the cross-sectional area of shrimp at its each segment (6-segments) using a computer vision system, developed by Luzuriaga *et al.* [25], suggesting two possible approximations: elliptical and circular approaches. The elliptical approach had its disadvantages since the eccentricity (a measure representing the roundness of an ellipse) varies through the length, bringing complexities in modelling the geometry. Therefore, the circular cross-section approach seemed to be an easier choice due to its compared simplicity to model whole shrimp geometry, where heat transfer can be assumed to fl w only in radial and longitudinal directions [10]. Then, applying this approach, shrimp can be approximated by a series of short cylinders with diminishing radii at each segment (Fig. 21.1). The volume elements that will be used in the numerical solution were then considered as a series of concentric rings stacked upon each other, with each consecutive set containing one less volume element representing the individual segments of shrimp. Based on this, the volume element network can be generated using the experimentally obtained dimensional data.

After composing the geometry, required equations for a given numerical scheme (e.g. finit difference methodology) are developed. For this purpose, two approaches can be used, truncated Taylor expansions or energy balance [26]. The energy balance method provides a better fl xibility, and capacitance and non-capacitance surface volume element approaches can be applied for handling surface boundary [26]. By performing an energy balance on each volume element, nine types of finit difference equations for different types of volume elements are developed. Chau and Gaffney [27] and Erdoğdu *et al.* [10] presented generation

of finit difference equations in detail. The following equations were for interior and outer side surface volume elements, respectively:

$$T_{i,j}^{n+1} = T_{i,j}^n + \frac{\Delta t}{(\rho \cdot c_p \cdot V)_{i,j}} \cdot$$
$$\times \left[\begin{array}{l} \dfrac{k_{i-1,j} \cdot A_{i-1,j}}{\Delta r_{i-1,j}} \cdot \left(T_{i-1,j}^n - T_{i,j}^n\right) + \dfrac{k_{i,j} \cdot A_{i,j}}{\Delta r_{i,j}} \cdot \left(T_{i+1,j}^n - T_{i,j}^n\right) \\[4mm] + \dfrac{k_{i,j-1} \cdot A_{i,j-1}'}{\Delta z_{i,j-1}} \cdot \left(T_{i,j-1}^n - T_{i,j}^n\right) + \dfrac{k_{i,j} \cdot A_{i,j}'}{\Delta z_{i,j}} \cdot \left(T_{i,j+1}^n - T_{i,j}^n\right) \end{array} \right] \tag{21.1}$$

$$T_{i,j}^{n+1} = T_{i,j}^n + \frac{\Delta t}{(\rho \cdot c_p \cdot V)_{i,j}} \cdot$$
$$\times \left[\begin{array}{l} \dfrac{k_{i-1,j} \cdot A_{i-1,j}}{\Delta r_{i-1,j}} \cdot \left(T_{i-1,j}^n - T_{i,j}^n\right) + \dfrac{k_{i,j-1} \cdot A_{i,j-1}'}{\Delta z_{i,j-1}} \cdot \left(T_{i,j-1}^n - T_{i,j}^n\right) \\[4mm] + \dfrac{k_{i,j} \cdot A_{i,j}'}{\Delta z_{i,j}} \cdot \left(T_{i,j+1}^n - T_{i,j}^n\right) + \dfrac{1}{\dfrac{1}{h \cdot A_{i,j}} + \dfrac{\Delta r_{i,j}}{(k \cdot A)_{i,j}}} \cdot \left(T_\infty - T_{i,j}^n\right) \end{array} \right] \tag{21.2}$$

After the finit difference equations are developed, explicit and implicit solution methodologies can be used to solve the resulting equations. Palazoğlu and Erdoğdu [28] presented explicit and implicit methods for solving finit difference equations. In the standard explicit method, firs the temperature for each volume element is calculated and used as the initial temperatures for the next time step.

To account for the shrinkage in the model, a dimensional shrinkage equation (obtained from experimental measurements) is required. Due to the temperature distribution inside shrimp as a result of the cooking process, each volume element can be assumed to have its own shrinkage value. In addition to dimensional changes, each volume element can also be accepted to have its own thermal and physical properties. Erdoğdu *et al*. [10] presented the following equations for variable thermal conductivity, specifi heat, and density of shrimp as a function of moisture content and temperature:

$$k = 0.0798 + 0.00517 \times (\%MC) \tag{21.3}$$
$$c_p = 1675 + 25 \times (\%MC) \tag{21.4}$$
$$\rho = 1029.9 + 0.6 \times T \tag{21.5}$$

where k is thermal conductivity (W/m-K), c_p is specifi heat capacity (J/kg-K), ρ is density (kg/m^3), T is temperature (°C), and %MC is the percent moisture content.

Thermal conductivity, specifi heat, and density are required parameters in modelling conduction heat transfer processes. These properties for different products and predictive methodologies can be found in the literature [29,30]. In addition to thermal and physical properties of shrimp, the convective heat transfer coefficien is another parameter required for modelling of heat transfer. It depends on thermo-physical properties of the heating/cooling medium, characteristics of food product (shape, dimensions, and surface roughness), and characteristics of flui fl w (velocity and turbulence) in the heat transfer medium [31].

For determining convective heat transfer coefficient there have been numerous expressions reported in the literature [32,33]. In addition, lumped system methodology and exact solutions for regular shaped geometries can also be used for this purpose [34].

The mathematical model, after determining the temperature distribution inside the product, can be expanded to predict the sterilisation values for safety consideration using inactivation kinetics (D- and z- values at a reference temperature) of a target organism:

$$F = \int_0^t 10^{\frac{T_c(t) - T_R}{z}} \cdot dt \tag{21.6}$$

where t is the processing time, $T_c(t)$ is the temperature change at the slowest heating point, T_R is the reference temperature, and z is the z-value (°C) of the given target micro-organism. Rippen and Hackney [35] reported the pathogen micro-organisms that might cause safety problems in seafood products with their inactivation kinetics.

Knowing temperature distribution also leads to calculated cook losses where each volume element can be assumed to have its own cook loss value as a function of cooking time ($t_{cooking}$) and the highest temperature (T_{max}) reached by that volume element during cooking [11]:

$$\%Cook\ Loss = f\ \left(T_{max}, t_{cooking}\right) \tag{21.7}$$

Since outer volume elements are subjected to higher temperatures for longer time periods than inner ones, higher cook losses are expected with a cook loss distribution throughout the product. Overall cook loss should then be calculated as the volume integral of the individual cook losses:

$$Cook\ Loss = \sum_{i=1}^{n} \sum_{j=1}^{l} \left[\frac{(m)_{i,j} \times (Cook\ Loss)_{i,j}}{m} \right] \tag{21.8}$$

where n and l are total number of volume elements in the radial and longitudinal directions, respectively; $(m)_{i,j}$ and $(Cook\ Loss)_{i,j}$ are the mass and cook loss value of the volume element (i,j), respectively; and m is the total mass.

Experimentally obtained predictive equations for cook losses are required to determine individual volume element cook losses [11]. Mass transfer, movement of water from inside to the outer surface, is not to be considered since mass transfer effects are indirectly addressed by including cooking time in the predictive cook loss equation (Eqn. 21.7). Zhang *et al*. [23] also assumed that the moisture losses would have a negligible impact on heat transfer although they represent a significan loss in weight. This might be due to the higher value of thermal diffusivity value compared to the moisture transfer, resulting in the temperature's coming into equilibrium quickly.

Results of mathematical models are definitel validated with experimental data. In some cases, exact solutions can also be used to validate the results of the numerical solutions. For conduction heat transfer, experimental model validation is easy since measurement of

temperature at a certain location is a simple task. In addition, in the case of the shrimp model, obtaining experimental cook losses can also be achieved with little effort.

21.5 Applications

In this part, use of a mathematical model for further cooking optimisation of shrimp is presented. General structure of the mathematical model for shrimp cooking is to predict the:

- temperature distribution for the given size of shrimp with known cooking temperature, convective heat transfer coefficient and experimentally obtained dimensional shrinkage;
- sterilisation value at the slowest heating point of shrimp using the previously calculated temperature distribution; and
- cook loss using the temperature distribution and experimentally obtained predictive equations.

Since the model could predict temperature distribution and cook loss during cooking, it might be easily used to develop shrimp cooking charts to optimise cooking parameters and establish cooking schedules [16]. Figure 21.2 shows a constructed cooking chart for large tiger shrimp (16–20/kg) based on the cook loss and various safety levels for a target micro-organism; *Vibrio cholera* ($D_{65°C} = 93$ s, z $= 7.7°$C). In this chart, cooking temperature was on the left *y*-axis while cooking time was on the *x*-axis and cook losses were on the right *y*-axis. The families of curves showed the desired reduction in the load of target micro-organism (1 log cycle to 9). The following example, adapted from Erdoğdu *et al.* [16], shows the use of this chart for cooking temperature and desired reduction of the given target micro-organism for an improved process design.
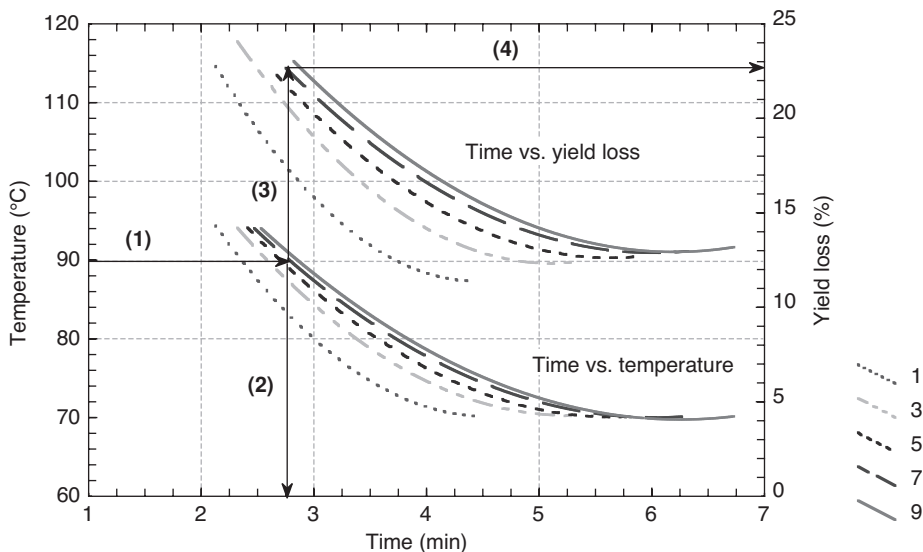

Fig. 21.2 A shrimp cooking chart constructed for large tiger shrimp (16–20/kg). Adapted from Erdoğdu *et al.* [16]. Copyright 2003, with permission from Elsevier.

Example (based on Fig. 21.2):

- **1st step** – cooking temperature: 90°C
- **2nd step** – cooking time: 2 min 40 s
- **3rd and 4th steps** – cook loss: 23% cook loss to obtain a 7 log cycle reduction in the number of target micro-organism.

21.6   Conclusions

Mathematical models are significan tools for process simulations and used for food process design and optimisation purposes. In thermal processing of seafood products, there are two important criteria, safety and quality (organoleptic and nutritional). In addition, cook losses are economical considerations. All these changes are directly affected by the temperature distribution of the product during thermal processing. Therefore, in this study, development of mathematical models for determining temperature distribution was explained, and incorporation of temperature distribution of the product for determining the sterilisation value (based on a pathogen micro-organism) and cook loss was shown. Using this information, construction of a shrimp cooking chart was presented to use the developed model for optimisation purposes suggesting an optimum thermal processing condition. As mentioned, besides safety and cook loss, retention of organoleptic and nutritional qualities might be additional constraints for process design. Similar approaches for determination of cook loss can also be incorporated in the models with known kinetic values of the different quality characteristics.

21.7   Nomenclature

$A_{i,j}$	Surface area of the volume element (i,j) in the radial direction $\left(2\pi r_{i,j}\Delta z_{i,j}\right)$	m^2
$AZ_{i,j}$	Cross-sectional area of the volume element (i,j) in the longitudinal area $\left(\pi\left(r_{i+1,j}^2 - r_{i,j}^2\right)\right)$	m^2
c_p	Specifi heat	J/kg-K
$\Delta r_{i,j}$	Distance between the volume elements in radial direction	m
$\Delta z_{i,j}$	Distance between the volume element in longitudinal direction	m
Δt	Time increment used in the explicit finit difference solution	s
h	Convective heat transfer coefficien	W/m^2-K
k	Thermal conductivity	W/m-K
l	Total number of volume elements in the longitudinal direction	
n	Total number of volume elements in the radial direction	
$r_{i,j}$	Radial distance from the centreline to the outer boundary of the volume element (i,j) in radial direction	m
ρ	Density	kg/m^3
T	Temperature	°C
T_∞	Heating/cooling medium temperature	°C
$T_{i,j}^n$	Temperature of volume element (i,j) at time step n	°C
$T_{i,j}^{n+1}$	Temperature of volume element (i,j) at time step n + 1	°C
$V_{i,j}$	Volume of the volume element (i,j)	m^3

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Plate 1 Irradiated salmon colors measured by Minolta and machine vision system and their actual pictures. Adapted with permission from Yagiz *et al.* [26]. This image also appears as Fig. 6.1.
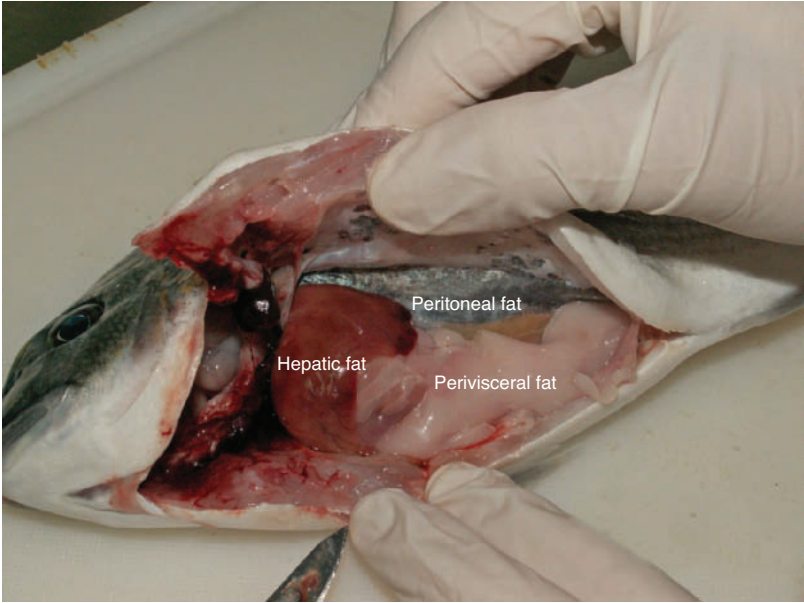
Plate 2 Anatomy of gilthead sea bream – forms of deposited fat, excluding muscle fat that is not visible. This image also appears as Fig. 7.1.

Glass container	**Metal containers**	**Paper products**
Flexible films	**Corrugated polypropylene box for carrying fish on board**	**IQF cooked shrimp packed in ring shaped thermoformed container**
IQF shrimps in flexible pouch	**Surimi**	**Thermoformed trays used for packing value added fish products**
Blanched breaded shrimp packed in thermoformed containers	**HDPE woven sack for bulk packaging of dry fish**	**Dried shrimp in consumer pack**
Tuna packed in polymer coated tin free steel can	**Ready to serve mackerel curry in retortable pouches**	**Fish sausages in casings**
Freeze dried products in pouches	**Freeze dried shrimps in cans**	**Fish pickle in flexible laminated pouches**

Plate 3 A range of packaging materials used for aquatic products. This image also appears as Fig. 12.1.

(a)

In vivo gene delivery system (IVGD)

DNA injection into spermatophore

Placement of electrode In spermatophore

(b)

Plate 4 Electroporation mediated *in vivo* Pacific white shrimp (*L. vannamei*) gene transfection. (a) Foreign DNA injection into spermatophore and placement of electrode into spermatophore of male shrimp. (b) Artificial insemination in Pacific white shrimp. Artificial insemination process: Male Pacific white shrimp with spermatophore extruded and sperm mass inserted into the fulcrum of female. Adapted from Chen *et al*. [23]. This image also appears as Fig. 22.1.
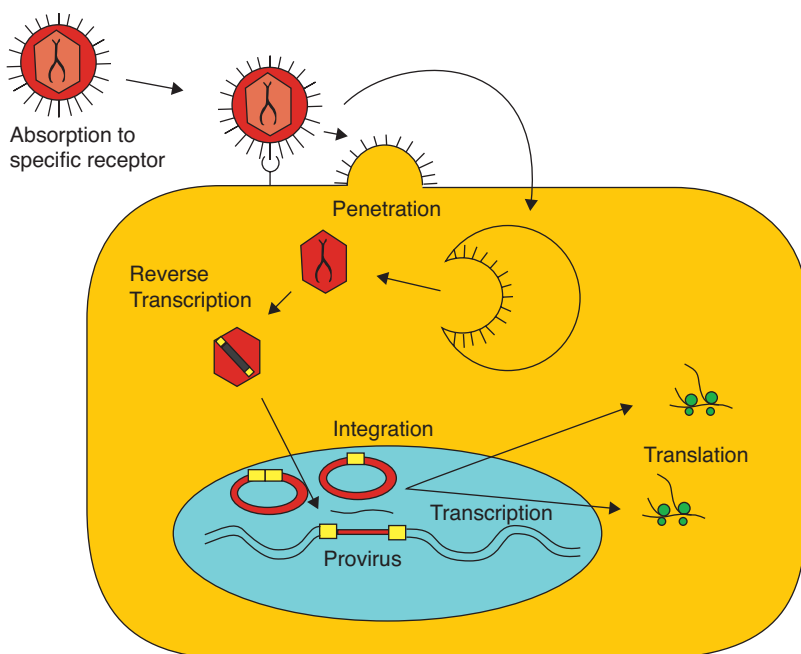
Plate 5 Methodology of gene transfer for aquatic species by recombinant retrovirus vector (one-time-only delivery system). Adapted from Lu *et al*. [16,18]. This image also appears as Fig. 22.2.
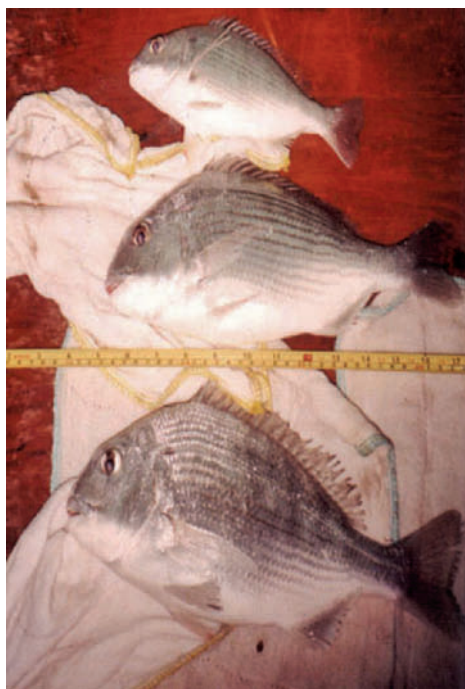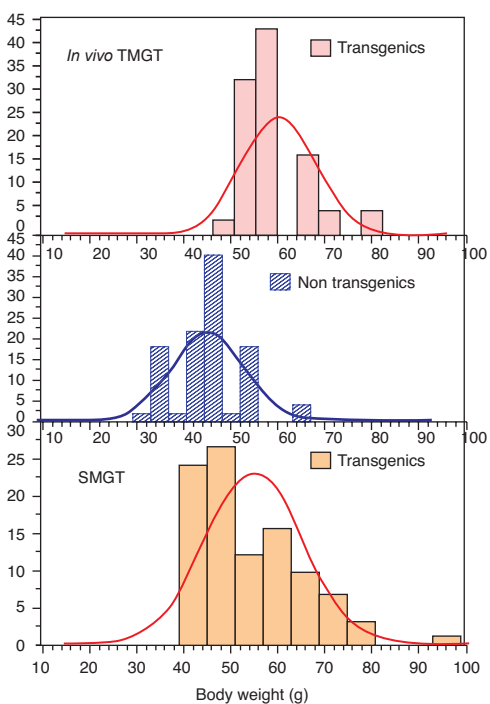
Plate 6 Growth performance of transgenic silver bream Po generation. Adapted from Lu *et al*. [22]. This image also appears as Fig. 22.3.

22 Transgenic/transgenic modified fish

Jenn-Kan Lu, Jen-Leih Wu, and Meng-Tsan Chiang

22.1 Introduction

Over the past decade, a revolutionary technique has been developed that allows the introduction of a define fragment of cloned DNA into germ lines of an animal. Once foreign DNA is integrated into a host genome, the DNA, now called the transgene, can be stably transmitted into progeny from generation to generation. Individuals carrying such transgenes are called transgenic animals and have been produced in various species such as *Caenorhabditis elegans* [1], *Drosophila* [2,3], sea urchin [4,5], *Xenopus* [6], mice [7–10], farm animals [11,12], and many fis and shellfis species [13–17].

Recent estimates by the United Nations indicate that the current supply of seafood products will have to increase seven-fold if we are to meet the worldwide requirement for fis and other seafood by the year 2020. Given the rapid decline in world fis stocks, caused mainly by over fishing it is clear that demand can only be met by aquaculture. Traditionally, the broodstock is selected based on cross-breeding to enhance the fishes beneficia traits. However, these traits are generally slow to emerge and unpredictable, and often the fis genome might not contain the gene mediating the desired effects. Transgenesis, therefore, holds promises for producing genetic improvements in fish such as enhanced growth rate, increased production efficien y, disease resistance, and expanded ecological ranges. In this chapter, some of these examples are highlighted. It is likely that transgenic fis might be the firs marketable transgenic animal for human consumption.

22.2 Methodology of gene transfer in fish

Fish have several advantages over mammals. Compared with mice, which produce few eggs, a spawning female fis can produce several dozen to several thousand eggs, providing a large number of genetically uniform materials for gene transfer manipulation. Once the gene transfer has been carried out using fis eggs, no further manipulation is necessary and the maintenance of the fis hatchery is relatively inexpensive. In contrast, fertilized mammalian eggs must be implanted into appropriately prepared recipient mothers, thus increasing the

preparative work and the operating cost. Several basic approaches for introducing foreign DNA into fis embryos have been developed.

22.2.1   Microinjection

Microinjection of cloned DNA fragments into pronuclei of fertilized mammalian eggs has been demonstrated as the most successful method for producing transgenic animals. However, the introduction of foreign genes into pronuclei of fertilized fis eggs is more difficul because the pronuclei are not readily visible, the egg has a tough chorion, and the perivitelline space is relatively large [18]. To date, most of the gene transfer studies in fis species using the microinjection method have been conducted by injecting foreign genes into the cytoplasm of an egg at the 1-, 2-, or 4-cell stage. Although these transgenes are integrated, expressed, and transmitted to subsequent generations in many transgenic fish the efficien y of foreign gene integration in these studies is low Chen *at al.* [19].

22.2.2   Electroporation

Instantaneous perturbation of cell membranes by sudden changes of electrical field has widely been used to introduce foreign genes into animal cells. Exposing eukaryotic cells to a brief but high voltage electric fiel can cause local areas of reversible membrane breakdown, allowing exchange of molecules through the transient "pores" in the membrane. Cells can be transformed to different phenotypes by exposing them to a high voltage, direct current (DC) electrical impulse in the presence of transgenes [20]. It has been demonstrated that this technique is effective in producing transgenic fis by directly exposing fis embryos to brief high voltage electric field [21–23]. However, the success rate was low and improvements and changes in the methodology are presently being pursued. Recently, we have developed several novel electroporation-mediated gene transfer methods: *in vivo* testis-mediated gene transfer (TMGT) and spermatophore-mediated gene transfer (SMGT) have been used to transfer foreign DNA into embryos of marine fis and penaeid shrimps (*Penaeus monodon* and *Litopenaeus vannamei*) for improvement of mass gene transfer techniques (Fig. 22.1).

22.2.3   Viral-mediated gene transfer (VMGT)

Methods have been developed utilizing retroviruses as vehicles for gene transfer in many species. One distinctive feature of retroviral vectors that makes them attractive candidates for use as agents for gene transfer is the life cycle of the retrovirus (Fig. 22.2). When the retrovirus penetrates a cell, the resultant viral RNA is firs converted to double stranded DNA. The DNA enters the nucleus and integrates into the host genome. The integration of viral DNA into the host is done through a site-specifi recombination reaction. An integration protein (IN) encoded by the retrovirus pol gene is required for this step. The provirus becomes part of the host genome, and the stability and maintenance of the transgene is far superior to that achieved using other gene transfer systems. Two limitations to the use of retroviral vectors are the restricted host-cell range and inability to produce a high-titer virus. A recombinant pseudotyped pantropic retrovirus derived from the Moloney murine leukemia virus (Mo-MLV) containing G glycoprotein of VSV (VSV-G) was used as the vehicle for the introduction of foreign genes into medaka embryos. It has been demonstrated that this recombinant retrovirus has an extremely broad host-cell range and can be concentrated in very high titre [24]. We have used electroporation to mediate retroviral vector penetration of

(a)
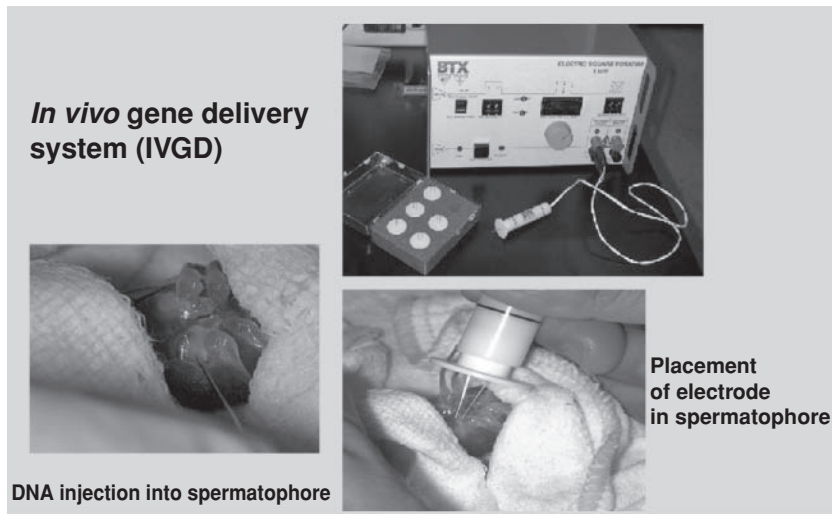
(b)
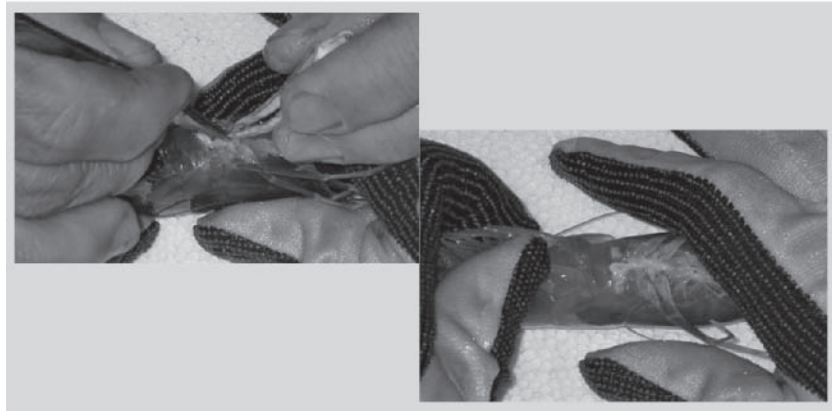
Fig. 22.1 Electroporation mediated *in vivo* Pacific white shrimp (*L. vannamei*) gene transfection. (a) Foreign DNA injection into spermatophore and placement of electrode into spermatophore of male shrimp. (b) Artificial insemination in Pacific white shrimp. Artificial insemination process: Male Pacific white shrimp with spermatophore extruded and sperm mass inserted into the fulcrum of female. Adapted from Chen *et al*. [23]. For a colour version of this figure, please see the colour plate section.

the vitelline layer of the fertilized clam egg [16,25]. Transgenic F_1 offspring were produced that contained a single, integrated copy of the provirus. This approach offers new possibilities for the phenotypic alteration of cultivated molluscs with introduction of genes for desirable traits such as disease resistance and accelerated growth.

22.2.4   The fate of the transgene

Regardless of the method of transfer, and the foreign DNA introduced into the developing embryo, it appears some transgenes might be integrated into the host genome of fis   tissues
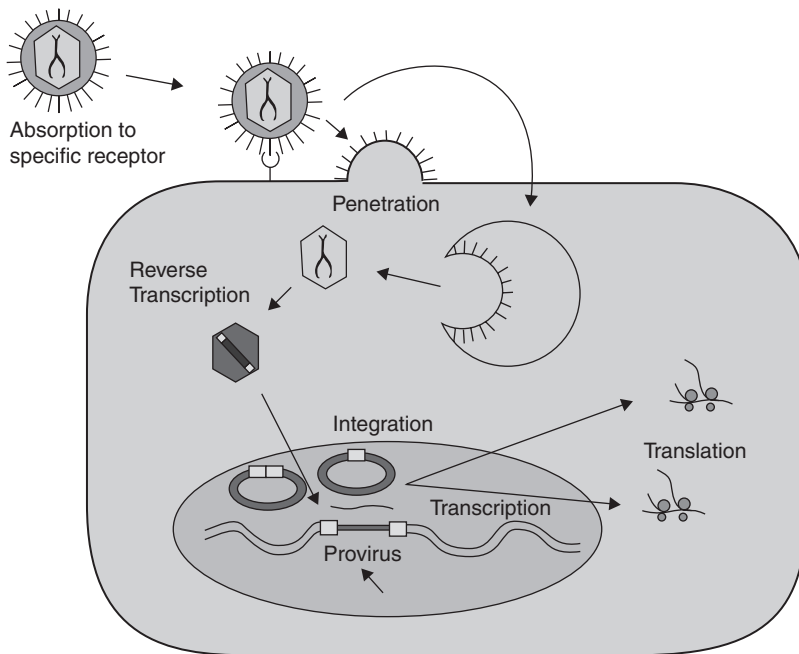
Fig. 22.2 Methodology of gene transfer for aquatic species by recombinant retrovirus vector (one-time-only delivery system). Adapted from Lu *et al*. [16,18]. For a colour version of this figure, please see the colour plate section.

and expressed in many tissues. The delayed integration causing mosaicism was very common and not all tissues contained the transgene and not all the cells within the transgenic tissues harboured the transgene. Copy numbers can range from one to several thousand at a single locus and, in contrast to the head-to-tail organization observed in the mouse system, in some but not all cases the DNA can also be found organized in all possible concatemeric forms, suggesting random ligation of the injected DNA prior to integration. Transgenes can integrate at single or multiple chromosomal locations for individual transgenic fish For our transgenic medaka and sea bream studies, the frequency of transgene transmission from founder animals was less than 50%, suggesting that integration of the foreign DNA occurs at later developmental stages. Transmission of transgenes to F_2 or later progeny occurs at Mendelian frequencies, indicating that the DNA is stably integrated into the host genome and passes normally through the germ line.

22.2.5 Why study gene transfer in aquatic animals?

Fish represent the largest and most diverse group of vertebrates and provide an advantageous system for studies of developmental processes. Fish are excellent candidates for the production of transgenics for several reasons. There are two important reasons for introducing novel genes into fis using gene transfer technology.

1) Conventional selective breeding of fis for improved growth or other characteristics is a very slow process. By contrast, the transgenic fis technology has the potential to improve genetic traits such as increased growth potential, disease resistance,

improved feed conversion efficien y, or other desirable genetic traits for aquaculture in one generation.

2) The production of transgenic fis can serve as an efficien approach for *in vivo* studies to gain knowledge of gene regulation and the action of gene products in vertebrates.

However, for application of the transgenic fis technology in aquaculture, there is a need to implement safeguards to prevent the introduction of these fis into the wild population. One solution is to produce sterile transgenic animals. The techniques for doing so, including whole genome manipulation, especially polyploid induction, gynogenesis, hybridization, and sex reversal, are fairly straightforward in fis species. The range of finfi species and transgenes used in this work is quite wide and the objectives of experiments to produce GM fis are shown in Box 22.1.

Box 22.1   Benefits expected from transgenic fish

Acceleration of fis growth
Increase of overall size
Higher yield of fille
Increase of food conversion efficien y
Superior utilization of carbohydrates as a low-cost diet
Cold tolerance
Freeze resistance
Control of sex and reproduction
Stress resistance
Improved sensory properties [colour, odour, fl vour, taste, and texture]
Improvement of nutritional properties

Because the increased prevalence of diseases and environmental stress have significantl reduced landings of some important fish/shellfi species for past decades, the production of farmed crustacean and molluscs is becoming increasingly important. Breeding programmes for crustacean and marine bivalve molluscs have lagged behind, when compared with terrestrial plants and animals. Broodstock for most bivalve species is usually collected from the wild. Therefore, genetic improvement by selective breeding cannot be conducted effectively because the genetic characters of the potential parents are unknown. However, few gene transfer techniques in bivalves and crustacean have been attempted [16].

 The increased prevalence of viral, bacterial, and other diseases has resulted in a severe reduction in shrimp production in a number of major producing countries. Gene transfer techniques offer a powerful approach for changing the genetic traits of economic crustacean species for both basic research and commercial applications. To date, only a few attempts have been made to produce transgenic crustacean species, with respect to disease-resistant aspects by using pantropic retroviral-mediated gene transfer (RMGT) techniques and electroporation mediated gene transfer methods [17; Lu & Chen, unpublished data].

22.2.6   Applications of gene transfer technique in aquaculture

Worldwide, more than 10 laboratories have reported the successful production of faster growing fis in several species. In the last decade, our laboratories and several others have
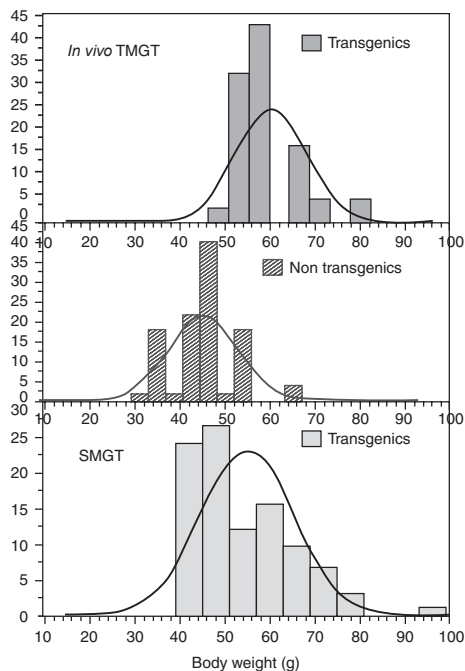
Fig. 22.3   Growth performance of transgenic silver bream Po generation. Adapted from Lu *et al*. [22]. For a colour version of this figure, please see the colour plate section.

successfully produced transgenic fis   containing a foreign growth hormone (GH) gene. The expression of the exogenous rtGH transgene is observed in faster growing rtGH transgenic fish and GH transgenic silver sea bream exhibit more than 50% of growth enhancement (Fig. 22.3). Furthermore, transgenic silver sea bream relative to control fis   exhibits a 10% improvement in gross feed conversion efficien y [22]. These studies demonstrate that the technology will be valuable to aquaculture. Shrimp is one of the most valuable seafood commodities, and the demand for crustacean is likely to grow in the future. Further growth of the shrimp aquaculture industry faces serious challenges. During the last decade, outbreak of epidemic shrimp diseases have resulted in large-scale mortalities in cultured shrimps. A major disease outbreak translates into loss of revenue and possibly the complete shutdown of operations. Several approaches are feasible using transgenic technologies. Antisense and siRNA technologies could be used to neutralize or destroy the viral RNA. Another possibility is to express the viral coat proteins, such as the WSSV in penaeid shrimp. The expression of this viral protein might titrate out the receptors for the virus, thus minimizing viral penetration. These two methods are effective but are restricted to related pathogens. We are currently testing antimicrobial peptide genes, such as cecropin from insect and monodocin from tiger shrimp, as candidates for gene transfer [Lu & Chen, unpublished data].

22.3   Food safety of transgenic fish

22.3.1   General concept

For evaluation of the food safety of growth hormone-transgenic fish the principal of "substantial equivalence" may be applied. The Organization for Economic Co-operation and

Development (OECD*)* group of National Experts of Safety in Biotechnology came to the conclusion "that no issue could be identifie which reduced or invalidated the application of the principle of substantial equivalence to food or food components derived from modern aquatic biotechnology" [26]. This means that wild or farmed fis of the same species should be used for comparison; fisher products made from non-transgenics. It was concluded that application of bio-engineering does not, in itself, result in a special risk to the consumer.

The general public has little understanding of the biology and the vagaries of how their food is grown and where it comes from, so public education of the positive and negative aspects of transgenic food and its risks is lacking [27]. Food safety issues posed by transgenic fis are discussed in the literature [28,29]. Concerns have been addressed of the possible risks of consumption of transgenes, their resulting protein, potential production of toxins by aquatic transgenic organisms, changes in the nutritional composition of foods, activation of viral sequences, and allergenicity of transgenic products. These risks have been analyzed, and while the majority of genetic modification to foodstuffs will be safe, the greatest potential for risk and harm is allergenicity.

This position was recently confirme by results from a safety consideration of DNA in food. It is clear that transgenic fis containing "all-fis gene cassettes" will not pose any risk to the consumer arising from uptake of the construct. Neither could special risks (e.g. by integration of DNA into human or gut microflor genomes) be identifie in instances where constructs consisted partly of non-fis elements. Uptake, degradation, and metabolism of substantial amounts (100–1000 mg per person/day) of foreign DNA are a normal process in human life. Other aspects to be regarded in respect of health risks for consumers are the gene product and the potential pleiotropic effect.

22.3.2   The gene product

Until now, in most instances of transgenic food fis production, the gene product has been a fis growth hormone, either from the same species (e.g. for tilapia) or from another fis species. In the case of the transgenic GH fish specifi experimental evidence that teleost GH is not active in primates was obtained by Guillen *et al*. [30]. Juvenile monkeys*, Macaque fascicularis* (macques) were injected with 1000 ng/kg of recombinant tilapia growth hormone per day for 30 days, equivalent to administering 70 μg/day to a 70 kg human. Guillen *et al*. [30] have shown that blood parameters examined included hemoglobin, serum total proteins, blood glucose, packed cell column, total leukocytes, and total erythrocytes. Body weight, rectal temperature, heart rate, and respiratory rate were recorded daily. Head to tail length, interscapular cutaneous pleat, left-flan cutaneous pleat, cranial circumference, and cranial diameter were measured. Tilapia GH did not affect animal behaviour pattern or food intake. Furthermore, body weight, temperature, heart rate, and respiratory rate were unaffected by tilapia GH administration to macques. The blood profile and somatic growth of tilapia GH treated macques and controls were not different. Autopsies revealed all organs, tissues, and cavities were normal, and no changes relative to controls were detected for common targets of GH such as tongue, palate plate concavity, liver, muscle, heart, kidneys, etc.

Guillen *et al*. [30] indicated that no histopathological or morphological changes were observed. Twenty-two humans were fed tilapia (transgenic hybrid *Oreochromis hornorum*) that contained and expressed tilapia GH transgene. This tilapia grew twice as fast as non-transgenic controls. The humans were fed transgenic or control tilapia for fi e consecutive days, twice daily. No clinical or biochemical parameters and no blood profile of humans evaluated before and after onset of experimentation were affected by consuming transgenic tilapia. The fact that tilapia (teleost GH) did not promote modification of blood glucose

values, total protein, and creatinine as well as having no affect on growth, target tissues, lipolysis, protein synthesis in the muscle, or any contra-insulin effects is indicative and confirm that fis GH is not bioactive in primates. Several studies [31,32] have shown that GH can stimulate erythropoiesis and lymphopoiesis and increase spleen and kidney weight and is associated with stimulating flui retention, growth, changes in blood volume, and blood characteristics, but none of these phenomenon were observed in transgenic fis studies. In addition, Dunham *et al*. [33] analyzed the theoretical food safety of GH transgenic carp. Levels of GH and insulin-like growth factors (IGF) expressed by transgenic GH salmon are not always outside the range or much greater than the upper limit of GH and IGF secretion for other fish food animals, or humans. Guillen *et al*. [30] also indicated that teleost fis GH and IGF are not bioavailable when orally ingested (cooked or raw, adequate cooking would denature the proteins) and even if they were totally bioavailable, the dose from one meal would only be a small fraction of total, daily, human production of GH and IGF. Growth hormone is not orally active in higher species, for example, fis GH is not bioactive in humans. The primate growth hormone receptor binds only primate growth hormone and it requires both binding sites to be occupied. The lack of oral activity of GH and IGF-I, and the nontoxic nature of the residues of these compounds, even at exaggerated doses, demonstrates that salmonid GH and IGF-I present no human safety concern when consumed orally. Thus, viewed from a number of aspects, any increased concentrations of GH or IGF in edible salmon skeletal muscle or skin is not hazardous to human health. A rGH transgenic silver sea bream study [22] indicated that no significan differences in body protein, dry matter, and ash were found in the F_1 transgenic silver sea bream relative to controls, while moisture content and lipid content were significantl lower. Our studies also have shown that the effects on the physiology, biochemistry, and histopathology of rat acute exposure to rGH transgenic F_1 silver sea bream fis meat administration indicated few effects on plasma glucose, plasma lipids levels, and on plasma insulin IgE and IgA concentrations (Table 22.1). No significan difference was also found in white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelet count (PLT) in rats after F_1 GH transgenic silver sea bream fis meat and non-transgenic sea bream diet oral administration. Few effects were found in liver function index, kidney function index, thiobarbituric acid reactive substances (TBARS) value, and blood lipid after F_1 GH transgenic silver sea bream fis meat diet administration (Table 22.1).

Although the concentration of growth hormone in organisms is low, much higher concentrations of other proteins, for example lysozyme or antifreeze proteins, are needed for proper fulfilmen of their function. Transgenic fis containing enhanced amounts of these proteins should be analyzed to determine the allergic properties of these proteins. These proteins occur naturally in several fis species but may not be expected in others. If there is any suggestion that they have allergenic potential, proper labelling of transgenic fis will be mandatory.

Foremost to many prospective consumers is the issue of food safety. Although cooking and digestion would break down most transgene products, three types of food safety concerns must be considered. First, bioactivity of the transgene product may pose concern, especially for pharmaceutical proteins. Second, allergenicity may prove hard to assess if the transgene comes from a non-food organism. Allergenicity assessment will be somewhat easier if the transgene comes from an organism representing known allergenic food groups, including fis and shellfish In this case, the transgene product can be tested for reactivity against antisera

Table 22.1 (a) Plasma glucose and plasma lipids levels in rats fed with different diets; (b) Effect of gene-modified fish meat on plasma insulin, IgE and IgA; and (c) Blood WBC, RBC, HGB, HCT, MCV, MCH, MCHC and PLT in rats after different diet oral administration

Plasma	A (CHOW)	B (*Sparus sarba*)	C (Gene-modified *Sparus sarba*)
(a)			
Glucose (mg/dL)	96.12 ± 26.50	90.71 ± 8.34	96.61 ± 9.29
Total cholestrol (mg/dL)	101.73 ± 12.90	89.57 ± 12.62	94.48 ± 3.81
Triacylglycerols (mg/dL)	31.39 ± 10.43[a]	25.81 ± 7.73	22.63 ± 4.51[a]
Phospholipid (mg/dL)	138.84 ± 13.3	137.62 ± 14.8	139.1 ± 13.7
TBARS (nmol/mL)	2.12 ± 0.89[a]	1.29 ± 0.13	1.07 ± 0.14[a]
GOT (U/L)	26.02 ± 11.5	24.62 ± 6.01	24.87 ± 4.89
GPT (U/L)	6.82 ± 1.40	5.71 ± 1.44	5.59 ± 2.19
Albumin (g/L)	33.59 ± 1.7	34.37 ± 1.24	34.10 ± 1.8
Urea acid (U/L)	0.31 ± 0.19	0.19 ± 0.07	0.18 ± 0.06
BUN (mg/dL)	8.65 ± 2.61[a]	13.3 ± 5.26[a]	12.99 ± 3.03
(b)			
Insulin (μU/mL)	2.62 ± 0.65	2.75 ± 0.70	2.53 ± 0.47
IgE (ng/mL)	27.74 ± 19.76	21.92 ± 16.31	20.89 ± 12.83
IgA (ng/mL)	245.88 ± 95.56	234.80 ± 98.28	242.77 ± 111.7
(c)			
WBC ($10^3/\mu L$)	6.54 ± 1.91	4.9 ± 1.07	5.1 ± 2.0
RBC ($10^6/\mu L$)	5.49 ± 0.39	5.63 ± 0.23	5.50 ± 0.36
HGB (g/dL)	11.77 ± 0.47	12.18 ± 0.42	12.15 ± 0.59
HCT (%)	32.10 ± 1.28	32.80 ± 0.85	32.68 ± 1.64
MCV (fL)	57.60 ± 4.67	58.31 ± 2.04	57.63 ± 1.34
MCH (pg)	21.49 ± 0.80	21.66 ± 0.89	21.43 ± 0.57
MCHC (g/dL)	36.67 ± 0.84	37.11 ± 0.65	37.21 ± 0.55
PLT ($10^3/\mu L$)	1144.83 ± 112.77	1126.00 ± 177.59	1075.40 ± 238.90

Data are expressed as means ± the standard deviations ($n = 6\sim8$). Means ± standard deviations followed by the sam letter, within a row, are not significantly different ($P > 0.05$).
Abbreviations: thiobarbituric acid reactive substances (TBARS), glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelet count (PLT).

from individuals with known food allergies. Third, toxicity potential is relatively easy to assess, and toxin genes would not be candidates for gene transfer.

22.4 Regulations of transgenic animals including aquatic animals

22.4.1 Environmental issues

In terms of ecology, there is no evidence that transgenics disrupt the ecological balance, and studies [22] suggest that transgenic fis harbouring a trout growth hormone gene are not different from non-transgenic controls. To avoid long-term environmental impacts, containment

of transgenic fis in culture situations is required and involves both physical and biological strategies. Obviously, sufficien physical containment is required to ensure that transgenic fis are not accidentally released. It is necessary to consider sterilizing fish the most practical way to biologically contain fis is through the use of hormone-treated or triploid sterilized populations.

22.4.2 Human health issues

Although most fisher resource managers agree that environmental issues are of primary importance, the human health concerns associated with genetically modifie organisms (GMOs) probably receive the most attention worldwide, probably as a result of news concerning genetically-modifie crops. Crops have been genetically modifie to retain pesticides, herbicides, and general antibiotics, and there are fears that these toxins could affect people [34]. Many of the GMOs being tested for use in aquaculture only produce more of their own growth hormone. Thus, from the human health perspective, the risks with the present use of the technology are clearly circumscribed and minor. One area of potential concern is the future development of disease resistance. A theoretical possibility is that, if a GMO is more disease-resistant, it may become a host for new pathogens, some of which may be transmissible or pathogenic to humans [35].

22.4.3 Trade

The Word Trade Organisation (WTO) agreements contain components that apply to GMOs (e.g. the removal of trade barriers, the requirements for intellectual property protection, and labelling requirements). Although no aquatic GMOs are traded, genetically modifie soybean is an ingredient of shrimp and other animal feeds that are traded globally. The European Community (EC) and Japan have labelling requirements for this feed, and the feed industry is studying the worldwide reaction to the labelling and may look for soybean replacements for feeds.

22.4.4 Intellectual property protection

The research, development, and production of reliable GMOs and the environmental and human health monitoring infrastructure that should be installed have financia implications for biotechnology companies promoting the use of GMOs. One mechanism to help recover these costs is through intellectual property rights, for example, patents that protect the inventors and developers of a product. Article 27(3)(b) of the Agreement on Trade-Related Aspects of Intellectual Property Rights (TRIPS) allows for the patenting of life forms. The US Patent Offic (1995, 1996) has granted patents on transgenic salmon and abalone. However, worldwide patenting laws are extremely complex and sometimes even contradictory. Although WTO and some countries allow the patenting of living organisms, the EC does not. Many groups have moral objections to the patenting of life (see the paragraph on Ethics below) and innovations that are contrary to public morality cannot be patented.

22.4.5 Labelling

The EC and the US are in conflic over the labelling of genetically modifie crops. Some countries maintain that labelling is impractical and would, in any case, be ambiguous while

others think that it is necessary for informed consumer choice and to prevent a public relations disaster. A major issue in labelling is that of "substantially equivalent", which means that if the GMO or product is equivalent to the non-GMO counterpart, no extra labelling is needed. How to assess equivalence, how much information should go on to a label, and how the authenticity of labels can be established, will be difficul matters to resolve [34].

22.4.6   Ethics

The fiel of ethics is extremely broad and ethics issues are often discussed under different terminologies. For example, some aspects of "responsible fisheries could also be referred to as "ethical fisheries" Ethical questions with regard to aquatic GMOs usually focus on whether humans have the right to modify natural creations.

22.4.7   Public perceptions

The potential economic benefit of transgenic technologies to aquaculture are obvious. Public acceptance of GMOs or products derived from them is likely to be a matter of education, demonstrations that they are safe to eat, approval by regulatory agencies, and the price and availability in the supermarkets. The DNA used in more recent studies including the promoters, such as the ocean pout AFP gene [36], the carp β-actin gene [37], and the genes (fis growth hormone, flounde AFP, and trout lysozyme), are all derived from the fis species. Since there are no toxin genes in the fis genome, Berkowitz and Kryspin-Sørensen [29] of the US Food and Drug Administration (FDA) have argued that there is no adverse effect on the insertion of DNA into the fis genome.

   Although there are theoretical causes for concern, there are no real data to support the recent claim that genetically modifie salmon are extremely dangerous to the environment. On the other hand, fis that have not been genetically modifie and that have escaped from culture facilities or have been introduced into environments outside their native range, have already caused environmental damage and are a clear and present danger. They have not received nearly so much press coverage. The most significan international action regarding GMOs is the establishment of the Secretariat of the Convention on Biological Diversity [38], a legally binding agreement under the Convention on Biological Diversity to protect the environment against risks posed by the transboundary transport of GMOs, which are similar to GMOs. Under this agreement, governments can decide whether or not to accept genetically modifie commodities, and commodities that may contain GMOs must be clearly labelled. When GMOs such as live fis are released into the environment, advanced informed agreement procedures must be followed, requiring that exporters provide detailed information to each importing country in advance of the firs shipment and that importers authorize shipments. However, pharmaceuticals produced by genetic engineering are not covered by the protocols. The relationship between protocols, which can restrict trade and existing WTO agreements [38] that aim for liberalized trade, need to be refined

22.5   Conclusions

Biotechnology provides powerful tools for the sustainable development of agriculture, fish eries, and forestry, as well as the food industry. When appropriately integrated with other technologies for the production of food, agricultural products, and services, biotechnology

can be of significan assistance in meeting the needs of an expanding and increasingly urbanized population in the 22nd century. Transgenic fis technology has provided a great potential in aquaculture industry. By introducing desirable genetic traits into finfi or shell-fish superior transgenic strains then can be produced for aquaculture purposes. These traits may include elevated growth rate, improved food conversion efficien y, resistance to some serious diseases, and tolerance to deteriorating environmental conditions.

In order to realize the full potential of the transgenic fis technology in aquaculture, several important breakthroughs are required:

1) developing more efficien mass gene transfer technologies;
2) identifying genes that have desirable traits for aquaculture purposes;
3) developing targeted gene transfer technology, such as the gene silencing technique;
4) identifying suitable promoters to direct the expression of transgenes at optimal levels during the desirable developmental stages;
5) determining optimal physiological, nutritional, and environmental factors that allow max-imized performance of the transgenic individuals;
6) assessing food safety and environmental impacts of the transgenic fish

Once the above problems are resolved, the commercial application of transgenic fis tech-nology will be readily attained.

Because several Asian and Pacifi economies are already world leaders in aquaculture, it seems both prudent and wise for those economies to focus a significan portion of their research on those areas of aquaculture biotechnology that will enhance their international competitive position in aquaculture and reduce the negative environmental impact that aqua-culture has on freshwater and coastal ecosystems.

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23 Molecular detection of pathogens in seafood

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23.1 Introduction

Seafood safety management may require information on the presence/level of pathogens at different stages of food chain. For example, microbiological criteria may be used to determine if shellfis can be harvested for raw consumption. The Food and Drug Administration (FDA) Guidance (Compliance Programme 7303.842) for *Vibrio parahaemolyticus* in ready to eat fisher products is at a level of 10^4/g (Kanagawa positive or negative); for some pathogens such as toxigenic *Vibrio cholerae* and *Listeria monocytogenes*, absence in 25 g. Detection or enumeration of pathogens by conventional microbiological methods takes several days. Rapid molecular methods are very useful for the food processing industry, food testing laboratories, national food inspection services, and for researchers. There is increasing emphasis on the use of risk analysis in seafood safety management. For assessment of risk associated with any microbial pathogen, data on the prevalence and concentration (level) of the pathogen at different stages in the food chain would be important and rapid molecular methods are very helpful in generating such data. In some organisms, such as *V. parahaemolyticus*, most environmental strains are not pathogenic to man and pathogenic strains are characterised by the presence of certain virulence genes, *tdh* encoding a thermostable direct haemolysin (TDH) or *trh* encoding a TDH-related haemolysin (TRH) [1]. Though TDH production can be detected in a high salt blood agar, wagatsuma agar, this test requires use of fresh rabbit or human blood, which is not easy to obtain in most food testing laboratories. There are no phenotypic tests for TRH production and molecular techniques are essential for their detection. This chapter deals with some of the molecular techniques commonly used in seafood testing or in research laboratories.

23.2 Probe hybridisation methods

Nucleic acid hybridisation methods have been described for a number of food-borne organisms. Probes binding to a variety of targets have been used, depending on the requirement. To detect a larger taxonomic group, probes binding to genes that are conserved in the group are used (Table 23.1), for example, ribosomal RNA gene probes for detection of *Salmonella*

Table 23.1 Comparison of application of traditional and molecular methods for seafood associated pathogens

| Pathogen | Application | Traditional method | | Molecular method | |
		Limitations	Advantages	Limitations	Advantages
Vibrio parahaemolyticus	Detection and enumeration	Takes several days, cannot detect some pathogenic strains (*trh+*), and strains with pandemic potential	Provides information about presence of viable bacteria	Some methods like PCR and real-time PCR are expensive and require skilled personnel	Rapid, can detect all known pathogenic and potentially pandemic strains
Vibrio vulnificus	Detection and enumeration	Takes several days	Provides information about presence of viable bacteria	Some methods like PCR and real-time PCR are expensive and require skilled personnel	Rapid
Vibrio cholerae	Detection	Takes several days, cannot detect toxigenic potential	Provides information about presence of viable bacteria	Expensive and require skilled personnel	Rapid, can detect toxigenic potential
Salmonella	Detection	Takes several days	Provides information about presence of viable bacteria	Expensive and require skilled personnel	Rapid, can detect strains showing biochemical variations (Lac+)
Listeria monocytogenes	Detection	Takes several days	Provides information about presence of viable bacteria	Expensive and require skilled personnel	Rapid
Noroviruses	Detection	Not available since these are not culturable in cell lines	Not applicable	Viability and infectivity can not be determined	Rapid, provides information on presence of viral nucleic acids
Haepatitis A virus	Detection	Takes several days	Provides information about presence of viable virus particles	Viability and infectivity can not be determined	Rapid, provides information on presence of viral nucleic acids
Fish-borne trematodes- *Clonorchis sinensis* and *Opisthorchis viverrini*	Detection	Cumbersome, requires high taxonomic expertise to differentiate liver flukes from other parasites	Detection of intact parasite stages, viability can be determined using experimental animals	Viability and infectivity can not be determined	Rapid, sensitive

spp. or *Listeria* spp. [2], *gyr*B gene encoding subunit B of DNA gyrase for detection of *Vibrio* spp. [3]. Probes binding to species-specifi genes, such as *Vibrio vulnificu* cytolysin gene, *vvh*A or *V. parahaemolyticus* thermolabile haemolysin gene, *tlh* or virulence associated gene coding for thermostable direct haemolysin, *tdh* [1,4], have been used for members of these species. The colony hybridisation technique has the advantage that it detects viable bacteria present in food, and colonies growing on non-selective agars can be specificall located for enumeration. Difficultie associated with use of radioactive probes have been overcome by the development of probes that are labelled with non-radioactive labels such as enzyme labels.

The FDA Bacteriological Analytical Manual (BAM) describes the colony hybridisation based method using alkaline phosphatase (ALP) labelled probes for enumeration of total *V. parahaemolyticus* and *tdh* + *V. parahaemolyticus* [1]. ALP labelled probes for enumeration of *trh* + *V. parahaemolyticus* have been described [5,6]. In this technique, homogenates of seafood are plated directly onto a T_1N_3 (1% tryptone and 3% NaCl) medium and the next day, the colonies are lifted onto filte paper, lysed, and subjected to probe hybridisation tests [1]. Thus, results are obtained in one day, compared to the four to seven days required to complete the traditional most probable number (MPN) method. Results obtained with the colony hybridisation method and BAM MPN methods are comparable [7]. However, for samples with very low *V. parahaemolyticus* densities, MPN is more sensitive (3 MPN/g) compared to direct plating and the colony hybridisation method (10 cfu/g). Spread plating on T_1N_3 after APW enrichment followed by colony hybridisation is superior to the conventional streak plate method for the recovery of pathogenic *V. parahaemolyticus* compared to the traditional streak plate method [8,9]. Digoxygenin labelled probes are simple to prepare in-house from polymerase chain reaction (PCR) amplifie fragments and have more reporter groups per probe molecule. When PCR fragments are labelled, twice the number of copies of the probe are prepared, since the reverse compliment is also labelled. However, hybridisation has to be performed on nylon membranes, while in the case of ALP labelled probes, inexpensive filte paper can be used [1].

ALP labelled probes binding to the cytolysin gene of *V. vulnificu* , *vvh*A are specifi and can be used for differentiating this species from other vibrios. FDA BAM [1] describes a method for enumeration of *V. vulnificu* using this probe for colony hybridisation following direct plating of seafood onto *V. vulnificu* agar (VVA). The colony hybridisation method and BAM MPN method for enumeration of *V. vulnificu* are in agreement more than 90% of the time, with the former being more rapid and precise, while the latter has lower limits of detection [10]. This probe can also be used in conjunction with non-selective agars [11], since direct plating on selective agars may underestimate *V. vulnificu* levels in environmental samples [12]. Enumeration of *V. vulnificu* and *V. parahaemolyticus* from water samples can be completed in one day using *vvh*A and *tlh* probe hybridisation of Hydrophobic Grid Membrane Filter (HGMF) colony lifts [13].

For enumeration of *L. monocytogenes* from seafood, DNA probe hybridisation methods in different formats have been described. The FDA BAM describes a colony hybridisation method using two probes, one designated AD07 binding to the *iap* gene encoding an invasion associated protein and another designated AD13 binding to the *hly* gene encoding the haemolysin and listeriolysin [2]. To avoid false negative reactions, use of both probes (designated AD713) has been recommended. Food samples homogenised in Listeria Enrichment Broth are spread plated on lithium chloride phenylethanol moxalactam (LPM) agar, and after 48 hours at 37 °C, the colonies from plates showing less than 300 colonies are lifted using Whatman 541 filters lysed, and hybridised with a radioactive AD713 probe [2].

DNA probe hybridisation based colorimetric assay *Listeria* GENE-TRAK has undergone validation and been adopted as the Officia Method 993.09 of the Association of Officia Analytical Chemists (AOAC) and has a sensitivity of 1 to 5 cfu/25 g [14]. The commercial Accuprobe is a test based on hybridisation of labelled probes to virulence factor mRNA, which ensures that only viable cells are detected [15].

The GENE-TRAK *Salmonella* assay is based on sandwich hybridisation and enzyme mediated colorimetric detection [16]. This dipstick assay involves capture and detector probes that are targeted to adjacent sequences of *Salmonella* ribosomal RNA. This assay is widely used and extensively validated with a sensitivity of 98.5%, which is equivalent of FDA BAM culture procedure, but with 3-stage enrichment, results are obtained in 48 hours [16]. This assay format has been further improved with the introduction of direct-labelled probe (DLP), for example, probe labelled at the 5′ end with horseradish peroxidase (HRP). In an AOAC Performance Tested Method (PTM) study, DLP assay showed 100% inclusivity (for all the serovars tested) and 98% sensitivity in food testing, and the commercial kit in microtitre format is available [16].

23.3   Nucleic acid amplification methods

23.3.1   Detection of bacterial pathogens

The most widely used nucleic acid amplificatio method in food microbiology is PCR, which can be compared to biological amplificatio (growth in culture) with enzymatic duplication and amplificatio of specifi nucleic acid sequences. The advantage of nucleic acid amplificatio methods over probe hybridisation methods is that very small numbers of target molecules can be enzymatically replicated in a short period of time to produce quantities of DNA that can be detected by a variety of methods. The sensitivity of most probes is around 10^4 to 10^5 target molecules and this makes hybridisation techniques unsuitable for direct detection of micro-organisms from food samples without enrichment or plating on agar media. For PCR, on the other hand, an average of less than 10 target molecules is sufficien to provide a positive result. The sensitivity can be further increased by targeting molecules that are present in multiple copies in a single cell, for example, rRNA sequences. PCR can be used to detect viruses that cannot be grown in cell culture. However, recovery of amplifiabl DNA from complex food matrices is a great challenge. Since dead cells contain amplifiabl DNA, a positive result obtained by using PCR does not conclusively demonstrate that viable organisms are present in a sample. To avoid such false positive results, it is advisable to draw a sample at two time points several hours apart. If the viable cells are present, the PCR yield from the second sample should be much higher because of the increase in the amount of template DNA. Alternatively, PCR could be performed after enrichment of samples in a broth.

PCR has been widely used for detection of several seafood associated pathogens (Table 23.2). While primers amplifying *tox*R have been used by most investigators for detection of total *V. parahaemolyticus*, few have used primers amplifying *gyr*B, *tlh*, and *vpm* gene encoding a metalloprotease or *V. parahaemolyticus* sequence in a recombinant plasmid pR72 H [17,18]. PCR amplificatio of *tox*R gene in lysates of enrichment broths at 6 hours detected *V. parahaemolyticus* in a larger number of samples compared to the conventional culture method [19]. PCR method based on amplificatio of the *tdh* gene for detection of pathogenic *V. parahaemolyticus* has been reported by several investigators [1,17,20]. Detection limit of more than 10^4 cfu/g when applied to lysates prepared directly from

Table 23.2 Summary of probe hybridisation based detection/enumeration methods

Organism	Method	Probe target	Applications	Regulatory agency approval	Reference
Vibrio parahaemolyticus	Radioactive or biotinylated or digoxygenin or alkaline phosphatase (ALP) labelled probes for southern blot or colony hybridisation	Various regions of tlh, tdh, and trh genes	Enumeration of total (tlh) and pathogenic V. parahaemolyticus (tdh and trh)	FDA method for enumeration by colony hybridisation by ALP labelled probes	[1,5,6]
Vibrio vulnificus	Radioactive or biotinylated or ALP labelled probes for southern blot or colony hybridisation	Various regions of vvhA gene	Identification of isolates or enumeration	FDA method for enumeration by colony hybridisation by ALP labelled probes	[1]
Salmonella	Sandwich hybridisation and enzyme mediated colorimetric detection	16S rRNA	Detection	AOAC Performance tested method	[16]
Listeria monocytogenes	Radioactive probe for colony hybridisation	iap and hly genes	Enumeration	FDA method	[2,14]
	Sandwich hybridisation and enzyme mediated colorimetric detection	16S rRNA	Detection	AOAC Performance tested method	

fis homogenates could be improved (<10 cfu/mL) by performing PCR after an 8 hour enrichment in alkaline peptone water [20]. PCR performed on lysates obtained from seafood homogenates without enrichment using primers amplifying *tox*R gene, yielded a higher number of positives compared to conventional isolation and PCR was more sensitive than colony hybridisation for detection of *tdh*+ and *trh*+ organisms [9]. Positivity increased with duration of enrichment, up to 18 hours. Studies performed using PCR on lysates from enrichment broths demonstrate that *trh*+ *V. parahaemolyticus* has a higher prevalence in the natural environment compared to *tdh*+ strains [21,22]. The FDA BAM recommends a multiplex PCR using primers amplifying *tlh*, *tdh*, and *trh* genes for identificatio and confirmatio of *V. parahaemolyticus* isolates and confirmin pathogenic potential [1]. This method is based on the work of Bej *et al*. [23], who noted a detection limit between 10^1 and 10^2 cfu/10 g when the assay was applied to seeded oysters that were pre-enriched for 6 hours.

The *V. parahemolyticus* O3:K6 serotype and a few other serotypes that are genetically similar have been incriminated in outbreaks in several countries and isolates have been referred to as pandemic strains [24]. Members of pandemic groups exhibit a unique sequence within the *toxRS* operon that encodes transmembrane proteins involved in the regulation of virulence associated genes and this has been used to develop group-specifi PCR (GS-PCR) differentiate pandemic and non-pandemic strains [25]. A unique open reading frame (ORF8) associated with a filamentou phage (f237) found in the genome of pandemic strain has been used as a target of PCR to characterise these strains [26]. While both of these PCR assays could detect O3:K6 pandemic strain and its serovariants, Chowdhury *et al*. [27] found ORF8 missing in 10% pandemic strains, and Okura *et al*. [28] found *tox*RS in a few *tdh*-strains. This suggests that neither of these tests can be used for a definit ve identificatio of pandemic strains.

For *V. vulnificu* , FDA BAM recommends PCR for confirmatio of isolates obtained by the MPN method [1]. Primers binding to different regions *vvh*A gene have been used [12] and nested PCR performed on enrichment broth lysates detected *V. vulnificu* in samples that were negative by colony hybridisation [11]. Nested PCR amplifying regions within the gene coding for 23SrRNA [29] could detect 10 fg of purifie *V. vulnificu* DNA that had been mixed with extracted eel DNA. However, these primers might miss some strains that are positive by *vvh*A PCR [30]. Kumar *et al*. [31] described a one-step PCR amplifying a fragment of *V. vulnificu* *gyr*B gene, which could detect the pathogen in enrichment broth lysates of oysters seeded with 30 cfu/g. Primers amplifying *vvh*A gene fragments have been used in multiplex PCR [32] for simultaneous detection of several food-borne pathogens (*Salmonella, V. cholerae, V. vulnificus* and *V. parahemolyticus*) at levels ranging from less than 10^1 to 10^2 cfu/g after 6 hours of enrichment [33].

Fish and seafood have been implicated in outbreaks of cholera in some countries, though there have been no cases associated with commercially processed seafood in the international trade [34]. *V. cholerae* is a highly heterogeneous species consisting of over 200 serotypes, of which only O1 and O139 are involved as causative agents of cholera. Non-O1/O139 serovars are widely distributed in the aquatic environments both in tropical and temperate parts of the world and these are not derived from faecal contamination of waters. Therefore, it would be important to differentiate choleragenic O1/O139 *V. cholerae* from other serovars. O1 and O139 antisera that are commonly used to identify choleragenic *V. cholerae* may cross-react with some environmental strains [34]. Choleragenic *V. cholerae* produce cholera toxin, which is the most important virulence factor of this organism. The *ctx* gene coding for the production of cholera toxin has been used by several investigators to design DNA probes

and PCR primers for specifi detection/identificatio of choleragenic *V. cholerae* [34]. The *ctx* gene is derived from a filamentou bacteriophage present in the genome of *V. cholerae* and sometimes, non-toxigenic O1 *V. cholerae* may be found in the aquatic environment and seafood [34]. Detection of choleragenic *V. cholerae* in fis homogenates containing less than 10 cells/mL is possible when PCR is performed after 6 hours of enrichment in alkaline peptone water [35]. FDA BAM recommends *ctx* based PCR for determining toxigenicity of *V. cholerae* [1].

Most regulatory agencies have zero tolerance for *Salmonella* in foods and verifying compliance with criteria such as absence of *Salmonella* in 25 g foods requires a highly sensitive technique for detection. Most PCR techniques described for *Salmonella* use enrichment before performing amplification Some of the early methods used gene targets such as genes encoding DNA binding proteins *hns* or *him*A and dot blot or Southern blot hybridisation for detection of amplificatio products and reported sensitivities ranging from 1 to 40 cfu/g in oysters, but several investigators have used agarose gels for detection of PCR products and target genes have varied, including *ori*C, *omp*C, *hns*, *inv*A, *uid*A, *spv*B, and random fragments [32,33,36,37]. However, lack of internal amplificatio control (IAC) in some of the assays, variation in detection limits, and accuracy and lack of validation using all the seven known subspecies of *Salmonella enterica* and *S. bongori* are some of the problems reported. In a multicentre evaluation of four primer pairs, primers amplifying the *inv*A gene had inclusivity of 96.6%, exclusivity of 100% and detected with a high probability of 5 to 50 cfu or 10 genomic copies of purifie *Salmonella* DNA per reaction in the presence of 30 to 300 copies of IAC [37]. This assay was evaluated in an inter-laboratory study using meat and environmental swabs that were subjected to pre-enrichment in buffered peptone water and cells lysed by thermal treatment and DNA extracted using Chelex –100 [38]. With meat samples, the assay showed accuracy of 99 to 100%, but with environmental samples, the accuracy was 91%. In fis samples, variations in results due to differences in enrichment broths could occur [36,39]. In another format, PCR-ELISA, amplificatio products are detected by probe hybridisation in microtitre plates and two commercial products for detection of *Salmonella* were based on this format [15].

PCR-based commercial BAX assay kit for *Salmonella* has undergone validation confirmin 98% sensitivity and has been adopted as AOAC Officia Method 2003.9 for various foods including fish Bennett *et al*. [40] reported that the limit of detection of the assay is 10^3 to 10^4 cfu/mL after enrichment. However, some false negative results, possibly due to PCR inhibitors in feed matrix, have been documented [41]. A multilaboratory study comparing Salmonella BAX with standard culture method in fi e food types including frozen fis (Tilapia) conducted by Silbernagel *et al*. [42], indicated that for all food types, at three inoculation levels tested, BAX system gave results comparable to standard culture method based on chi-square results.

L. monocytogenes is another pathogen of great concern in seafood safety management and currently many countries have zero tolerance for this organism in ready to eat foods such as smoked fish A number of PCR based methods have been described for the detection of *L. monocytogenes* and the target genes include 16S rRNA, 23S rRNA, 16 S-23S intergenic space region, genes coding for virulence associated proteins listeriolysin (*hly*), internalin (*inl*A, *inl*B), actin polymerisation (*act*A), phospholipase (*plc*A, *plc*B), metalloprotease (*mpl*) regulator of virulence genes (*prf* A), genes coding for other proteins such as aminopeptidase, Sigma B factor, delayed hypersensitivity protein, and *L. monocytogenes* antigen [15]. False negative results due to PCR inhibitory factors in food matrix have been a major impediment for the use of PCR for direct detection of *L. monocytogenes* in foods. Most PCR protocols

involve selective enrichment for 24 to 48 hours before DNA extraction and some methods involve use of magnetic beads, dipsticks, or membranes to separate DNA from PCR inhibitors [15]. The commercial BAX *L. monocytogenes* method, adopted as AOAC Officia method 2003.12 for various foods including seafood involves PCR followed by probe hybridisation in a microtitre plate [15].

23.3.2   Detection of viral pathogens

Among the two most important viruses associated with seafoods (particularly bivalve molluscs), noroviruses, and hepatitis A virus, the former cannot be grown in cell cultures and the latter is slow growing, requiring about three weeks to show growth. Wild-type strains are difficul to culture and may not show cytopathic effects, and techniques such as immunoflu orescence have to be used to detect growth. The infectious dose of food-borne viruses could be small and ingestion of even 10 to 100 viral particles has a high probability of causing an infection [43]. Unlike clinical samples, where viruses are present in large numbers, foods may carry a small number of viruses and detection of these small numbers in a complex food matrix has been a great challenge.

Most methods to detect viruses in foods involve an elution/concentration step before extraction of nucleic acids. A variety of buffers have been used for elution and an additional adsorption-elution (adsorption to solid surfaces facilitated by lowering pH, conductivity, and followed by elution in buffers) procedure has been used in some cases. Recovery efficien y of 48% for poliovirus in oyster tissue [44] could be improved to 60% by modificatio of buffers using adsorption elution method. Polyethylene glycol 6000 (PEG 6000) or PEG 8000 has been used for precipitation of viruses and magnetic poly(dT) beads for purificatio of viral poly (A) RNA [44]. Extraction of nucleic acids from concentrated samples involves enzymatic digestion (e.g. proteinase K) and phenol/chloroform extraction or guanidinium thiocyanate extraction followed by adsorption to solid silica substrate [44,45].

Both norovirus and Hepatitis A virus are RNA viruses and reverse transcription PCR (RT PCR) used for their detection involves synthesis of complementary DNA (cDNA), which is then amplifie by PCR. Noroviruses are divided into fi e genogroups (GG), GGI- GGV of which GGI and GGII are most commonly involved in human infections. The ORF1-ORF2 junction region is highly conserved and has been the target for primers in several studies, including those involving shellfis [45]. For detection of Hepatitis A virus, real-time RT-PCR assays targeting 5′ untranslated region (5′ UTR) are sensitive and specifi [46]. Viruses have been commonly detected in commercially produced shellfis by PCR [47] but it is not clear whether this represents presence of infectious viruses. Currently virus detection is not included as a part of regulatory monitoring of shellfis in most countries.

23.3.3   Detection of parasites

Fish-borne parasites are a cause of public health concern and it is estimated that over 18 million people are infected with fish-born trematodes [48]. Detection of trematode metacercariae in fis by microscopy is a laborious process involving digestion of fis tissue, sedimentation of parasites, and observation of concentrated material. Often several parasites are found in fis and differentiation of metacercarial stages of liver flu es from that of intestinal trematodes requires a high level of taxonomic expertise. PCR techniques have been described for detection of *Opisthorchis veverrini* and *Clonorchis sinesis* in fis and snails [49–52]. In the case of *C. sinensis*, the assay could detect DNA equivalent to 0.46 metacercaria (51). Primers described for *O. veverrini* were specifi for this species while tested using *O. felinius* DNA and in spiked fis tissue, could detect three metacercariae [52].

23.3.4   Real-time PCR assays

Real-time PCR (also called quantitative PCR or qPCR) is now becoming popular for the detection of food-borne pathogens due to the possibility of quantitating them and eliminating the need for gel-based detection of PCR products. There are two methods of quantifying the PCR products:

1) use of fluorescen dyes that intercalate with double stranded DNA; and
2) use of modifie oligonucleotide probes that fluoresc when hybridised with complementary DNA.

Dyes such as SYBR Green that bind dsDNA would bind to all dsDNA, including non-specifi products or primer dimers, but are less expensive and can be used for any target to be amplified The fluorescen reporter probes, on the other hand, need to be specificall synthesised for each reaction. The TaqMan assay is an example of this, where a single stranded oligonucleotide probe complementary to a segment of 20 to 60 nucleotides within the DNA template and located between the two primers is used. In this assay, a fluorescen reporter (e.g. 6-carboxyfluorescin and quencher (e.g. tetramethylrhodamine) are covalently attached to the 5′ and 3′ ends of the probe, respectively. The single stranded probe does not show fluorescenc due to close proximity of fluorochrom and quencher. During PCR, the 5′ to 3′ exonuclease activity of Taq polymerase degrades the portion of the probe that has annealed to the template, releasing the fluorochrom from proximity to the quencher. Thus fluorescenc is directly proportional to the fluorophor released and amount of DNA template present in the PCR product. With both types of assays, the exponential increase in fluorescenc is used to determine the cycle threshold (Ct), which is the number of PCR cycles at which significan exponential increase in fluorescenc is detected. Using a standard curve for Ct values at different DNA concentrations, quantitation of target DNA in any sample can be made.

Real-time PCR targeting *tdh* gene using TaqMan format was more sensitive compared to streak plate/probe method for detection of pathogenic *V. parahaemolyticus* in enrichments of naturally contaminated oysters [53]. Multiplex real-time PCR targeting *tlh*, *tdh*, and *trh* genes of *V. parahaemolyticus* using different TaqMan probe labels has been used to investigate an outbreak associated with contaminated mussels [54]. Correlation between Ct and $\log_{10}$ number *V. parahaemolyticus* in shellfis in real-time TaqMan PCR targeting *tlh* gene [55] and *tox*R gene [56] is well documented. TaqMan real-time PCR assay targeting gyrB gene had a detection limit of 6 to 8 cfu per reaction in spiked oyster [57]. TaqMan multiplex real-time PCR targeting *tlh*, ORF8, *tdh*, and *trh* genes detected total and pathogenic *V. parahaemolyticus* with detection limits of 1 cfu/g oyster after overnight enrichment [58]. Tyagi *et al*. [59] reported SYBR green-based real-time PCR assay targeting *tdh* gene, which had a detection limit of 1 cfu/mL in shrimp homogenates after 6 hours of enrichment.

For *V. vulnificu* , Campbell and Wright [60] found TaqMan real-time PCR assay cytolysin gene to have a sensitivity similar to colony hybridisation using ALP labelled *vvh*A (designated VVAP) gene probe. SYBR green-based assay of Panicker *et al*. [61] targeting cytolysin gene had a detection limit of 10^2 cfu/g of oyster tissue homogenate and 10^2 cfu/mL seawater and the sensitivity could be increased to 1 cfu/g with 5 hours enrichment. Molecular typing techniques such as restriction fragment length polymorphism (RFLP) are useful in differentiating clinical and environmental isolates of *V. vulnificu* [62]. Real-time PCR assay designed based on the sequence variations in the 16S rDNA could be used for detection and differentiation of

clinical and environmental strains without isolating them, with 82% agreement between typing by RFLP and real-time PCR assay [63].

The qPCR for detection of toxigenic *V. cholerae* in spiked foods, including shrimp and oysters, has been reported using the *ctx*A gene as the target [64]. The detection frequency was 87% by culture and 98% by qPCR after 6 hours of enrichment and 83% by culture and 100% by qPCR after 18 hours of enrichment. In an 8 hour working day, toxigenic *V. cholerae* could be detected after 6 hours of enrichment when the initial levels were 1 to 2 cfu/g.

Though application of real-time PCR for detection of *Salmonella* in foods has been reported, efficacy in naturally contaminated fish or seafood is not well established. The Taqman assay using primers and probes within the ttrRSBCA locus, which is located near the *Salmonella* pathogenicity island 2, correctly identified 110 *Salmonella* strains tested and the detection probability was 100% when a suspension of 10^4 cfu/mL was used and 70% with suspension of 10^3 cfu/mL, though 20 fish fillet samples tested were negative by both traditional culture methods (ISO 6579:2003) and real-time PCR assay [65]. Real-time PCR targeting *inv*A gene for detection of *Salmonella* in shrimp and other food matrices had a sensitivity of 0.04 cfu/g [66]. A method that uses fluorescence resonance energy transfer (FRET) between two probes that hybridise to adjacent sequences on the amplified DNA sequence has been described by some investigators. Two fluorochromes are used here, one at the 3′ end of the first probe and another at the 5′ end of the second probe. When excited by the external light, the first fluorochrome transfers energy to the second flurochrome which then emits light at a specific wavelength and a LightCycler is used in this method [16]. A detection limit of 10 cfu/reaction has been reported by Perelle *et al*. [67] for fish samples enriched in buffered peptone water for 18 hours before DNA extraction.

L. monocytogenes detection in various foods by real-time PCR has been evaluated by several investigators [68]. An assay involving 4 hours of enrichment in Fraser broth followed by DNA extraction, real-time PCR targeting *ssr*A gene of *L. monocytogenes* had a detection limit of 1 to 5 cfu/25 g food sample and the assay could be performed in 2 working days compared to up to 7 days by the standard ISO 11290-1 culture method [69]. Compared to the standard method, the specificity was 99.4% and sensitivity 96.15%. With 42 fish samples, one smoked salmon produced false negative result and one fish swab produced false positive result. The sample showing the false negative result showed positive reaction for other *Listeria* spp. [69].

23.3.5   DNA microarray assays

The use of DNA microarray technology for the study of food-borne pathogens has been attempted by some investigators. Some of the approaches used include PCR amplification and hybridisation with microarray. The targets for PCR include one more universal genes such as 16S rRNA or 23S rRNA, or pathogen-specific genes such as virulence associated genes or random or arbitrary fragments or genomic probes selected by comparative genomics [70]. Studies related to seafood are not common and the complexity and cost of the assay limits its application in routine seafood testing.

23.4   Conclusions

The applications, limitations, and advantages of molecular techniques in comparison to traditional methods have been summarised in Table 23.3. The list of molecular methods that have undergone inter-laboratory calibration, validation, and approval by regulatory agencies

Table 23.3 Gene targets for PCR based detection of bacterial pathogens associated with seafoods

Organism	rRNA genes	Virulence associated genes	Genes coding for regulatory proteins	Genes coding for enzymes	Other genes	Reference
Vibrio parahaemolyticus	16S RNA	tdh and trh	toxR and toxRS	gyrB	tlh, pR72H, and ORF8	[1,17,20]
Vibrio vulnificus	23S RNA	vvhA		gyrB		[31]
Vibrio cholerae	–	ctx	toxR	–	–	[1,35]
Salmonella	16S rRNA	pvB and invA	hns and himA	uidA	ompC and oriC	[32,33,36,37]
Listeria monocytogenes	16S RNA 23S RNA 16S-23S RNA intergenic spacer region	Hly, inlA, inlB, iap, actA, plcA, plcB, and mpl	prfA and sigB	aminopeptidase	Dth and fbp	[2,15]

is growing. Consequently, they are being used in testing for compliance with microbiological criteria or for validation of process control or HACCP measures. Scientifi data collected using molecular techniques would be helpful for assessing the risk due to pathogens in foods. For example, the FDA quantitative risk assessment on the public health impact of *V. parahaemolyticus* in raw oysters uses a model in which the ratio between pathogenic and total *V. parahaemolyticus* in oysters was determined based on data obtained using molecular tools [71]. The need to consider pathogenic rather than total *V. parahaemolyticus* in seafoods has been pointed out by the European Commission [72]. With increasing application of risk assessment in taking food safety management decisions, the need for data on pathogens would increase and molecular techniques will have an important role in generating such data.

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24 DNA-based detection of commercial fish species

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24.1 Introduction

International growth in the trade and consumption of fis has led to an increased potential for species substitution or mislabelling [1]. This fraudulent practice is illegal in both domestic and global markets and has numerous detrimental consequences. For example, mislabelling of an endangered species could lead to market exploitation and interfere with fisherie conservation and management programmes. Alternatively, mislabelling fis species could expose consumers unknowingly to health risks that are associated with certain species, such as allergens or toxins. Fish species substitution can also be a form of economic deception, where a species of lower quality is mislabelled as a higher-quality species that commands a greater market price (Table 24.1). In all cases, the occurrence of species substitution can lead to consumer mistrust and confusion, ultimately resulting in a general avoidance of fis products. Therefore, the ability to regulate fis species substitution is essential to ensuring public confidenc and trust in the food supply.

Previously, the majority of species identificatio methods were based on protein analysis, such as isoelectric focusing (IEF) and immunoassays; however, DNA-based methods are growing in popularity due to their increased specificit , sensitivity, and ability to be recovered from heavily processed food products [2]. Within the fiel of DNA-based species identification there are numerous gene targets and detection methods available, each with its own advantages and disadvantages [3]. For the most part, research groups have thus far worked independently to develop methods for detection of fis species groups of commercial interest. While it is beneficia to have a variety of techniques and gene targets available, there is currently a lack of standardized protocols for fis species identification Improving the coordination of research efforts will greatly facilitate progress in this rapidly growing discipline and help standardize DNA-based fis and seafood species identification This chapter covers a brief discussion of current DNA-based techniques and gene targets, followed by an examination of major collaborative research efforts working towards DNA-based fis species identification

Table 24.1 Examples of seafood substitution cases that have been known to occur. Adapted with permission from Rasmussen & Morrissey [3]

True identity	Mislabelled as	Potential economic gain (US$/kg)[a]
Rockfish	Red snapper	5.42–6.00
Yellowtail	Mahi mahi	na[b]
Mako shark	Sword fish	na
Alaska pollock	Cod	0.62–3.35
Sea bass	Halibut	0.71–1.79
Arrowtooth flounder	Dover sole	0.66
Paddle fish and other fish roe	Caviar (sturgeon species)	>1000[c]
Steelhead trout	Salmon	up to 3.02
Farm-raised salmon	Wild salmon	up to 1.74
Pink salmon	Chum salmon	0.37
Imported crabmeat	Blue crabmeat	na

[a]Potential economic gain is calculated as the difference in average ex-vessel prices (US landings 2006, price per pound) between the two species groups listed [61].
[b]na, not available.
[c]According to 2008 retail prices.

24.2 DNA-based methods and gene targets

24.2.1 DNA-based methods

The major DNA-based methods that have been used in fis species detection include: forensically informative nucleotide sequencing (FINS); restriction fragment length polymorphism (RFLP); random amplifie polymorphic DNA (RAPD); amplifie fragment length polymorphism (AFLP); single-stranded conformational polymorphism (SSCP); species-specifi multiplex polymerase chain reaction (PCR); and real-time PCR. The basis for each technique is briefl discussed and compared in this section (Table 24.2) and examples of laboratory results for the different methods are given (Fig. 24.1; [4–9]). Several review articles discussing these methods have been published [3,10,11].

Sequencing-based identificatio techniques, such as PCR-FINS, are the most direct and reliable route to obtaining species-specifi information; however, they are also relatively time-consuming and cannot be used on mixed-species samples [12,13]. In order to differentiate species with this method, a gene target must be used that exhibits high inter-species and low intra-species variations. Following PCR amplificatio and sequencing of the gene target, the nucleotide sequence is compared to a set of reference sequences. Species can then be identifie by determining which reference sequences exhibit the lowest genetic distance to the target sequence. This method has been utilized to identify numerous fis and seafood species, including snapper, rockfish and tilapia [14], small pelagic fis used in canned sardine products [15], and octopus and squids [8,16].

PCR-RFLP is one of the most widely used techniques for fis species detection to date [17]. With this method, a pair of universal primers is employed to amplify the same DNA region in all species. The resulting amplicon is then digested with restriction enzymes that recognize and cut short sequences of DNA. In order to differentiate species, enzymes are chosen that target regions of variation between species, and the result is a species-specifi pattern of restriction fragments that can be detected with gel electrophoresis or lab-on-a-chip capillary electrophoresis. This method is low-cost and relatively simple, but can be

Table 24.2 Comparison of major DNA-based methods used in fish and seafood species identification for prevention of commercial fraud. Adapted with permission from Rasmussen & Morrissey [3]

DNA-based method	Acronym or alias	Requires prior DNA sequence information?	Quantity of loci analysed	Robustness to DNA degradation	Potential for inter-laboratory reproducibility	Cost	Potential for database construction	Potential for intraspecies variation errors	Examples of fish and seafood species identified with method
Species-specific primers and multiplex PCR	n/a	Yes	Single	Medium-High	High	Medium	High	Medium	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, sharks, molluscs
DNA sequencing + phylogenetic mapping	FINS	Yes	Single	Medium-High	High	High	High	Low	Cephalopods, gadiformes, molluscs
Restriction fragment length polymorphism	RFLP	Yes	Single	Medium-High	High	Medium	Medium-High	Medium	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, molluscs
Single-stranded conformational polymorphism	SSCP	Yes	Single	Medium-High	Medium	Medium	Medium-High	Low-Medium	Salmonids, scombroids, sturgeon, eels
Random amplified polymorphic DNA	RAPD	No	Multiple	Low-Medium	Low-Medium	Medium	Medium-High	Low-Medium	Percoids, goosefish, molluscs
Amplified fragment length polymorphism	AFLP	No	Multiple	Low-Medium	Medium-High	Medium High	Medium-High	Low-Medium	Salmonids, scombroids

time-consuming, as it requires a post-PCR restriction digest that may be as long as overnight. PCR-RFLP assays have been developed for the detection of a wide range of commercial species, including flatfi [18], white fis [19], scombroids [20], and snappers [21].

In PCR-RAPD, a short PCR primer is designed to amplify random fragments throughout the genome of the target species [22]. The result is a species-specifi DNA band profil that can be visualized with gel electrophoresis. This method is relatively low-cost and easy to carry out; however, some problems have been reported in terms of reproducibility and interpretation of results. PCR-RAPD assays have been developed for the differentiation of percoids [23], mussels [7], and abalone [24].

PCR-AFLP is a technique that involves aspects of both PCR-RFLP and PCR-RAPD. Prior to PCR, two restriction enzymes are digested with genomic DNA. The resulting DNA fragments are then amplifie with PCR primers that anneal to adaptor molecules bound to the restriction sites. Two selective PCR steps are carried out in order to reduce the total number of amplifie fragments to about 100, and then the species-specifi DNA profil is obtained using gel electrophoresis. This method has shown higher reproducibility than PCR-RAPD and involves relatively low start-up costs; however, it is time-consuming and requires the use of high-quality DNA, which may prove challenging in the case of processed food products. While PCR-AFLP has experienced limited use in fis species identification it has been used to differentiate two salmonid species [9] and several tuna and bass species [25].

PCR-SSCP is a highly sensitive species identificatio method that has proven to be more robust against intraspecies variation as compared to RFLP and RAPD [26]. However, it is also more demanding than other methods and commands a high level of reproducibility. With PCR-SSCP, a specifi gene target is amplifie and then denatured into single-stranded DNA. The denatured DNA is then analyzed with polyacrylamide gel electrophoresis (PAGE). Differences in mobility through the gel network reflec variations in nucleotide sequences between samples, thereby allowing for species detection. PCR-SSCP assays have been developed to identify numerous fis and seafood species, including salmonids [27], scombroids [4], fis from multiple species groups [28], and clams [29].

Whereas many techniques utilize universal primers to amplify the same gene target from a wide range of species, species-specifi primers are designed to anneal to regions of DNA that are conserved within a species and are variable between species [12]. This method is advantageous in terms of its low cost and speed, as the results can be detected by gel electrophoresis immediately following PCR. Furthermore, species-specifi primers can be combined in a single tube in a multiplex PCR assay, thereby reducing preparation time and materials. On the other hand, the development of these methods requires extensive background research and laboratory optimization. Species-specifi and multiplex PCR assays have been utilized to detect a variety of fis species, including gadoids [30], scombroids [31,32], sturgeons [33], and sharks [34]. Multiplex PCR can be adapted for use with real-time PCR, in which fluorescenc is used to detect the target DNA fragments. During PCR, a fluorescen signal indicates amplificatio of the target DNA, allowing for rapid species detection in real time. Real-time assays have been developed to differentiate fis species such as cod, haddock, and whiting [35], perches and groupers [36], and tuna [37].

24.2.2   Gene targets

Selection of an appropriate gene target for species identificatio involves consideration of several factors, including the integrity and origin of the starting material, the range of species to be differentiated, and the detection method [3]. In the case of fis species detection, it is
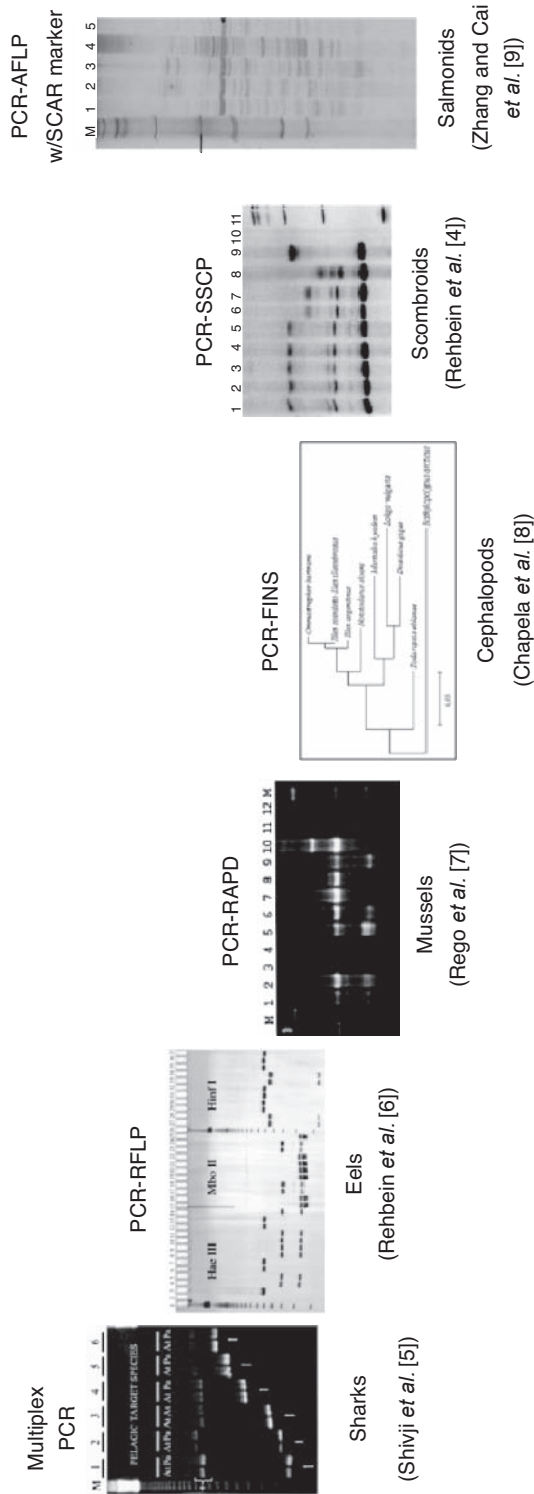
Fig. 24.1 Examples of laboratory results of DNA-based methods used for the identification of fish and seafood species. Adapted with permission from Rasmussen & Morrissey [3].

generally desirable to choose a relatively short gene target that can be expected to survive food processing and shows sufficien variation between species. Due to its simplicity, high copy number and relatively fast rate of mutation, mitochondrial DNA (mtDNA) has been widely used to identify species in fis and seafood products. On the other hand, nuclear DNA is more complex, exhibiting features such as heterozygous alleles, introns, repetitive DNA, and transposable elements. Some specifi mtDNA regions that have been targeted for species identificatio include the genes coding for cytochrome *b*, cytochrome *c* oxidase subunit I (COI), 12S ribosomal ribonucleic acid (rRNA), and 16S rRNA. These gene targets have been used for differentiation of a great number of species groups, including sturgeons, flatfis codfish salmonids, gadoids, scombroids, eel, cephalopods, crabs, and many others [17]. Furthermore, the use of cytochrome *b* and COI as standardized species-level markers has been extensively researched and both have been shown to be effective at discriminating closely related species. The use of these gene targets in major collaborative research efforts will be discussed in the following sections.

Despite the widespread use of mtDNA, nuclear DNA targets have also proven to be advantageous for some instances of species identification For example, nuclear DNA can be used to identify species that are known to hybridize, as opposed to mtDNA, which is maternally inherited. Some nuclear gene targets that have been utilized in fis species identificatio include 5S rRNA, the nuclear ribosomal internal transcribed spacer 2 (*ITS2*), and microsatellite DNA [17].

24.3   Major collaborative efforts

Fish species substitution is a worldwide problem, both in terms of domestic and international trade. Furthermore, due to increased global trade of fis products, species that were previously only consumed in one geographical region are now exported worldwide, thus increasing the range of species that must be differentiated. In order to facilitate progress in this field collaborative efforts to develop and standardize appropriate methods for identifying species substitution should be carried out on an international scale. Two examples of such efforts are discussed here: FishTrace and the Fish Barcode of Life Initiative (FISH-BOL).

24.3.1   FishTrace

The FishTrace Consortium is a collaborative effort consisting of 10 member institutions spanning 7 European countries. The project is funded by the European Commission (EC) and its aim is "to catalyse the cooperation and the pooling of data and material corresponding to the genetic identificatio and characterization of marine fis species from European waters and marketed in Europe" (http://www.fishtrace.o g). Thus far, over 200 commercial fis species from 8 different European sea regions have been sampled and analyzed as part of the FishTrace project. For each species, detailed information is available regarding its morphological and biological features, the original specimens sampled and analyzed, and the nucleotide sequences for two genetic targets: cytochrome *b* (mtDNA) and rhodopsin (nuclear DNA). Sequence information is given for the entire cytochrome *b* gene (1141 bp) and for a portion of the rhodopsin gene (460 bp). The use of these two gene targets was reported to improve the efficien y of species identification as they are from different genomic locations and exhibit different rates of evolution [38]. In order to facilitate high-throughput analysis, a set of 21 PCR primers was developed that allows for the amplificatio of both cytochrome *b* and rhodopsin in over 200 teleost fishe from 17 Actinopterigii orders [38].

Twelve of the primers were designed to amplify the cytochrome *b* gene and 9 primers are specifi for rhodopsin. Although the entire cytochrome *b* gene can be amplifie with one reaction, the authors suggested two separate reactions to improve amplificatio efficien y. Furthermore, a nested or semi-nested PCR step was necessary in most cases in order to obtain DNA of sufficien quality for automated sequencing. Overall, Sevilla *et al*. [38] reported this protocol to be a powerful tool for sequencing and species identificatio of teleost fishes

24.3.2 DNA barcoding

The Consortium for the Barcode of Life (CBOL) is an international collaborative effort focused on establishing DNA barcoding as a global standard for species identificatio (http://barcoding.si.edu/). CBOL consists of over 160 member institutions from more than 50 countries in such diverse geographical regions as Africa, East Asia, Eurasia, Oceania, and North, South, and Central America. The results of this project are intended to facilitate molecular species identification the discovery of previously unrecognized species, biodiversity research, and development of a handheld DNA-based identificatio device [39]. DNA barcoding is based on the use of a short, diagnostic nucleotide sequence to assign unknown individuals to a species group, and research efforts have been focused on an approximately 650 bp region of COI as the standard DNA barcode [40,41]. Although protein-coding mtDNA genes in general are good targets for species identification COI was determined to be advantageous as a DNA barcode due to its high phylogenetic signal and the existence of robust universal primers that enable recovery of a diagnostic gene fragment from most, if not all, animal phyla. Also, because changes in its amino acid sequence occur more slowly than with cytochrome *b*, COI was determined to have a greater potential to provide deeper phylogenetic insights [40].

24.3.2.1 *DNA barcoding of fish*

FISH-BOL was established in partnership with CBOL and represents a global effort to establish a reference sequence library of DNA barcodes obtained from voucher specimens for all fis species (*www.fishbol.o g*) [42]. It is the intention of FISH-BOL to complement existing sources of information, such as FishBase and genomics databases. Similar to the FishTrace project, for each species posted on FISH-BOL, detailed information is provided on the nucleotide sequence and the voucher specimens, along with important links to taxonomic and biological information.

Thus far, barcode sequences for over 7,000 fis species have been obtained and numerous studies have been published on the use of DNA barcoding for fis species identification These studies are discussed in the following section, along with the potential use of DNA barcoding to detect fis species substitution.

Ward *et al*. [43] sequenced the COI barcode region in 207 species of Australian marine fish 143 species of teleosts and 61 species of sharks and rays. In order to account for intraspecies diversity, multiple specimens were sequenced for most species (average 3.66 individuals/species). Kimura two parameter (K2P) distances, which are a measure of nucleotide sequence divergence, averaged 0.39% within species, 9.93% within genus, and 15.46% within family, and all species could be differentiated by DNA barcoding. Interestingly, members of the *Thunnus* genus showed low intra-genus variation (1.11%) due to their recent divergence as compared to other species; however, species discrimination was still possible through barcoding. In a subsequent study, the authors expanded upon the initial sample collection to include a total of 388 species of fishes 4 Holocephali, 61 Elasmobranchii,

and 323 Actinopterygii [44]. Each species was represented by one individual. All species could be differentiated with DNA barcodes, except for two that are suspected to hybridize, the stingarees *Urolophus cruciatus* and *Urolophus sufflavu* . As expected, the nucleotide sequence diversity of COI was found to be much greater than the COI amino acid sequence diversity, with a high ratio of synonymous to non-synonymous mutations. That is, even though the COI nucleotide sequence shows extensive variation between species, the protein itself remains relatively conserved. In another study into Australian fishes Pegg *et al*. [45] examined the utility of DNA barcoding to identify fis larvae in the southern Great Barrier Reef of Australia. Out of three primer sets tested, the best results were found with the primers reported previously by Ward *et al*. [43]. The authors found that DNA barcoding allowed for successful identificatio of all species tested, including those from the commercially important genera *Plectropomus*, *Epinephelus*, *Lethrinis*, and *Lutjanus*.

The North Pacifi Ocean and the Bering Sea are home to 15 species of skate from 3 genera (*Amblyraja*, *Bathyraja*, and *Raja*: Rajidae) [46]. These skate are especially vulnerable to exploitation as a by-catch in other directed fisheries however, management has been complicated by uncertainty surrounding species composition. The potential for DNA barcoding to assist with fisherie management and conservation in the identificatio of Alaska skate species was therefore investigated [46]. A 498 bp fragment of COI was sequenced in 1 to 9 individuals from each species, and 13 out of the 15 species examined were found to have unique sequences allowing for species identification Based on these results, the authors found potential for the use of DNA barcoding to complement traditional methods for species identificatio of skate. However, there is a need to sequence more specimens across the species ranges in order to fully determine fi ed and polymorphic differences.

Rock *et al*. [47] examined the use of DNA barcoding to identify fis species common to the Scotia Sea, Antarctica. DNA barcodes were obtained for 35 putative fis species from 9 families. For each species, 1 to 12 individuals were sequenced. The results showed no effect of geographical sampling on sequence variation and there was strong congruence with field based morphological identifications DNA barcoding was able to differentiate two families of fis (Liparidae and Zoarcidae) that could not be resolved morphologically. However, species within the genera *Bathydraco* and *Artedidraco* could not be discriminated based on nucleotide sequences from both COI and cytochrome *b*, indicating possible hybridization events and haplotype sharing. Overall, it was found that DNA barcoding could be a valuable tool for fisherie and ecological monitoring in the Scotia Sea.

The use of DNA barcodes to identify Canadian freshwater fis in the context of FISH-BOL was examined by Hubert *et al*. [48]. COI barcode sequences were collected from 1,360 individuals representing 190 species from 85 genera and 28 families. K2P distances were fairly similar to those found previously for marine fish averaging 0.3% within species and 8.3% within genera. In most cases, the COI sequences from different species formed tight clusters in distinct groupings, allowing species identificatio through DNA barcoding. However, 13 species exhibited shared or overlapping haplotypes with sister species, possibly due to hybridization. There were also a few cases of deep divergences between two individuals of the same species, which may be resolved by a re-examination of the current taxonomy of these species groups. Based on the overall results of this study, DNA barcoding was determined to be a powerful method for the identificatio of most Canadian freshwater fis species.

24.3.2.2 *DNA barcoding for the detection of fish species substitution*

DNA barcoding has numerous potential regulatory and forensic applications in the fis industry, including fisherie monitoring, controlling trade of endangered species,

enforcement of fis product traceability, and detection of species substitution in the commercial marketplace [49]. In this respect, the COI gene has been assessed for potential use in forensic analysis with specimens of cow, chicken, and fis (cod, *Gadus morhua*) [50]. Species-diagnostic COI sequences were obtained in all validation experiments. In cases where misidentificatio did occur, it was attributed to either a lack of primer specificit or due to erroneous reference sequences. Overall, it was determined that COI can be used to consistently identify species provided that a data base of authenticated reference sequences is available.

The US Food and Drug Administration (FDA) has examined the potential use of DNA barcodes in the *Regulatory Fish Encyclopaedia* (RFE), which is an on-line resource used to identify fis species [51]. The RFE contains detailed identificatio information for 94 species of commercial fish however, thus far species-specifi DNA profile have not been posted to assist with species identification With the intention of updating the RFE with DNA barcodes, 72 species of fis from 27 families were sequenced at the COI barcode region. After the barcodes were obtained, a blind study was carried out with 60 unknown fis muscle samples. Based on the reference DNA barcodes, all 60 samples were correctly identifie at the species level, and DNA barcoding was determined to be a valuable method for species identification The FDA is now hoping to expand the barcode data base to encompass all fis species that may enter the commercial market. Furthermore, DNA barcoding has already been applied for regulatory purposes by the FDA and the Chicago Department of Public Health to identify a toxic puffer fis species that was the source of a food poisoning event in Chicago, IL [52]. The puffer fis had been illegally imported as monkfis and the results of DNA barcoding were used as evidence in the investigation, which led to a recall of 282 mislabelled products that had been distributed to three states.

The use of DNA barcoding to identify species in commercial fis products was recently tested in the North American marketplace [53]. A total of 96 samples of fis and seafood muscle tissue were acquired from commercial markets and restaurants in Canada and the US. Ninety-one samples were successfully amplifie and 90 of those were identifie at the species level, with sequence matches of more than 97%. Based on the barcoding results, 23 of the samples were suspected to be mislabelled. Out of a total of nine red snapper (*Lutjanus campechanus*) samples that were purchased in New York City, seven were determined to be mislabelled as one of fi e other fis species. In another example of mislabelling, a sample sold as white tuna sushi (*Thunnus alalunga*) was identifie to be Mozambique tilapia (*Oreochromis mossambicus*). These results illustrate the utility of DNA barcoding as a regulatory tool for species identificatio in commercial fis products.

Work has also been ongoing to create a DNA barcode data base for the identificatio of commercially important marine species in New Zealand waters [54]. Close to 400 out of a total of 1,200 fis species found in the New Zealand Exclusive Economic Zone have been barcoded [55]. Based on the barcode data base, species assignment was possible for 24 suspect shark fillet that had been confiscate by New Zealand fisher officers DNA barcoding was also utilized to identify species in smoked fis fillet from a variety of species, including longfi eel (*Anguilla dieffenbachii*), snapper (*Pagrus auratus*), blue cod (*Parapercis colias*), and hoki (*Macruronus novaezelandiae*) [56]. A pair of universal primers was used to amplify the barcode region in all samples, which included fis species from 10 families and 4 orders (Salmoniformes, Anguilliformes, Gadiformes, and Perciformes). Species was identifie at the 99 to 100% level for all samples and the authors predicted that as the data base of barcode sequences expands, DNA barcoding will become a standard method for identificatio of fis species in food products.

24.3.2.2.1 Mini-barcodes

Although DNA barcoding has proven successful for the identificatio of a range of commercial fis species, it may prove challenging to obtain a full barcode ($\sim$650 bp) from heavily processed products that contain degraded DNA. In such cases, the use of a "mini-barcode" ($\sim$100–200 bp) has been proposed [57]. Indeed, based on a bioinformatics analysis, Meusnier *et al*. [58] found that while the full-length DNA barcode allows for the maximum species identificatio (97% species resolution), a 250 bp fragment was predicted to allow for species identificatio in 95% of cases, and a 100 bp fragment was predicted to identify species in 90% of cases. Universal mini-barcode primers were developed to amplify a 130 bp fragment of the COI barcode. These primers were tested with DNA extracts of over 1,500 specimens from 691 species of mammals, fishes birds, and insects. With a PCR success rate of 92%, amplificatio of the mini-barcodes proved to be more successful than amplificatio of the full-length barcode for all but one species group (e.g. insects from the order Plecoptera). The use of mini-barcodes with commercial fis products has yet to be tested; however, it is likely that these smaller DNA fragments will prove useful for species identificatio in heavily processed food products, such as canned fish

24.3.2.2.2 COI-based multiplex PCR assays

As an alternative to a sequencing-based assay, a few studies have developed COI-based multiplex PCR assays for rapid species identificatio in commercial fis products. As mentioned previously, these assays are advantageous in that they can be used with mixed-species samples and do not require access to sequencing equipment. Marshall *et al*. [59] developed a multiplex PCR assay for forensic discrimination of the sea scallop (*Placopecten magellanicus*) and the Icelandic scallop (*Chalmys islandica*). Although both scallop species are harvested commercially in the North Atlantic Ocean, there was concern that sea scallops from a closed fisher were being retained illegally as by-catch in the Icelandic scallop fisher . Multiplex PCR was utilized to identify species in 967 scallops seized from two fishin vessels and the test results in both cases revealed extensive sea scallop poaching. In another example, a multiplex PCR assay was developed for the differentiation of fi e *Crassostrea* oyster species common in China [60]. Successful identificatio was achieved using a single PCR tube and the method was reported to be simple, fast, and reliable.

24.4 Conclusions

A variety of DNA-based techniques have been applied to the detection of commercial fis species substitution, including traditional sequencing, FINS, RFLP, RAPD, SSCP, AFLP, species-specifi multiplex PCR, and real-time PCR. As discussed, each of these methods has its own advantages and disadvantages, and selection of the appropriate method and gene target for species identificatio involves a careful evaluation of factors such as the quality of the starting material (e.g. degree of processing) and the number of species that must be differentiated. In order to standardize fis species identificatio on an international level, major collaborative efforts have been initiated, such as the FishTrace project and FISH-BOL. The FishTrace project is focused on genetic identificatio through cytochrome *b* and rhodopsin sequences, whereas FISH-BOL is based on COI sequences. Both approaches will prove valuable to the identificatio of commercial fis species. As demonstrated by the numerous publications involving COI barcodes, there is strong potential for DNA barcoding in regulatory and forensic applications for the detection of commercial species substitution and illegal

fisherie operations. However, DNA barcoding does have some limitations, including the inability to identify inter-species hybrids and mixed-species samples. In cases where species boundaries are blurred by hybridization or haplotype sharing, the use of a nuclear gene will be necessary for species differentiation. Samples suspected to contain multiple species will need to be identifie using alternative techniques, such as the multiplex PCR assays described above. Overall, this fiel will greatly benefi from coordinated efforts for the development of a variety of standardized, complementary methods for DNA-based species identification

24.5 Acknowledgements

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25 Seafoods and environmental contaminants

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25.1 Introduction

Seafood is one of the major sources of protein and functional components that are crucial to human health and is an important part of the diet in many countries. On the other hand, seafood usually contains residues of persistent environmental pollutants (PEPs), such as organohalogen compounds: polychlorodibenzodioxins (PCDDs) and polychlorodibenzofurans (PCDFs) namely dioxins, polychlorinated biphenyls (PCBs), polychlorinated naphthalenes (PCNs), polybrominated diphenyl ethers (PBDEs), organochlorine pesticides (OCPs), and heavy metals (mercury-Hg, arsenic-As, cadmium-Cd, and lead-Pb). Dietary intake is the major contributor of human exposure to these contaminants and seafood has the greatest risk due to its high lipid content. These lipophilic chemicals accumulate in fatty tissues of marine organisms and are subsequently transferred to humans through the food chain, damaging ecosystems and human health.

PEPs in seafood have gained special attention from consumers, scientists, and environmental and governmental organizations due to their widespread consumption. Several studies have determined PEPs in aquatic organisms from freshwater and marine environments. Beside the marine ecosystem, products from aquaculture are also associated with increased risk of contamination by these pollutants, as well as antibiotic residues. This chapter discusses the wide range of studies on seafood contaminants and the factors behind the contamination. Furthermore, regulations for these contaminants and recommendations to reduce the risk of contamination are provided.

25.2 Persistent environmental pollutants (PEPs)

25.2.1 Organohalogen compounds (OCs)

OCs is a group of chemically stable, carbon-based, and ubiquitous compounds characterized by low volatility and high lipid solubility. They are formed mainly through combustion, waste incineration, metal recycling and refining chemical manufacturing, biological, and

photochemical processes [1]. OCs can enter aquatic environments from industrial wastes, fis farm activities, agricultural cultivations, and untreated sewage discharge, as well as from the rinsing of the soils through fl ws and shipping traffic They then adsorb onto suspended particulates and are deposited on the sediments [2]. Aquatic organisms accumulate these compounds in their tissues through direct contact with the water, suspended particles, and bottom sediments, as well as through their diet, thus enabling the assessment of transfer of pollutants through the trophic web and biomagnification [3]. These compounds can be dispersed into the environment and reach remote regions via currents, streams, and migratory organisms. The presence of these pollutants in the tissues of Antarctic organisms is a confirmatio of this [4].

For the general population, dietary intake contributes up to 90% of human exposure to OCs [5]. The levels of these compounds in blood [6] or mother's milk [7] provide a reliable marker of human exposure to these compounds. Many market researches in Spain [8], Belgium [9], and the US [10] stated that fis and fis products, and those with high fat content in particular, exhibit higher OCs levels than any other foodstuff. Among OCs, PCBs, PCDDs, PCDFs, PCNs, PBDEs, and OCPs are well studied for risk assessment.

25.2.1.1   *Dioxin and dioxin-like compounds*

Dioxin is a generic name of PCDFs and PCDDs, mainly the by-products of industrial and natural processes. Waste incineration is the largest contributor to the release of PCDDs and PCDFs into the environment, particularly if combustion is incomplete [11,12]. PCDDs, PCDFs, and their brominated analogues (PBDDs/PBDFs) include 75 and 135 congeners, respectively. Only 17 congeners of PCDDs/PCDFs and PBDDs/PBDFs exhibit dioxin-like toxicity. On the other hand, 12 of the 209 PCB congeners are referred to as "dioxin-like" PCBs as they exhibit biological activity similar to that of PCDD/PCDFs [13]. Due to the complex nature of the congeners, seven congeners (IUPAC no. 28, 52, 101, 118, 138, 153, and 180) have been chosen as indicators on the basis of their persistence in the food web and tendency to biomagnificatio [11]. Among them, PCB-138 and -153 are the most abundant congeners. For the risk evaluation of dioxin-like compounds (DLCs) in humans, fish and wildlife, which are commonly found as complex mixtures, the concept of toxic equivalency factors (TEFs) has been introduced. TEFs compare the potential toxicity of each compound in the mixture to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD), which is the most toxic congener, classifie as a group I carcinogen with a TEF of 1.0. TEF values can be used to calculate toxic equivalent (TEQ) concentrations and these can be used as a relative measure between different abiotic and biotic samples [13].

Kiviranta *et al*. [14] determined the PCDD/PCDFs and PCBs in 1,573 herring samples collected from the Baltic Sea. The concentrations of PCDD/PCDFs and PCBs were in the range of 6 to 67 pg/g and 9.4 to 490 pg/g wet weight (ww), respectively, which showed an increased tendency with age and size of samples. Schröter-Kermani *et al*. [15] determined the PCDD/PCDFs concentrations of bream samples collected from rivers of Germany and reported higher values than the maximum permitted value set by the European Commission (EC) (4 pg WHO-TEQ/g ww) for three sampling sites. In the Gulf of Naples, Italy, Naso *et al*. [16] found the sum of 20 PCB congeners relative high in edible tissues of sea bass (22,286.6 ng/g lipid weight-lw). Similarly, the sum of 18 PCB congeners was highest in sea bass collected from Antifer, France. However, at the same sampling location, the PCDD/PCDFs concentration was highest in mussels [17]. In the North Sea, Belgium, Σ PCB

ranged between 1.5 and 280 ng/g (ww) among benthic invertebrates. The highest range was reported as 650 to 3,200 ng/g (ww) in the liver of the gadoid fish bib [18]. Perugini *et al*. [19] reported anchovy, mackerel, and pilchard as the most polluted species from Italian coasts of the Adriatic Sea in terms of PCBs.

25.2.1.2 *Brominated flame retardants (BFRs)*

BFRs are synthetic compounds that have been widely used to decrease the likelihood and intensity of fir in a variety of consumer products. Several classes of compounds have been used, such as polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBP A), and hexabromocyclododecane (HBCD) [20]. To date, the majority of environmental studies have focused on PBDEs. There are 209 different PBDE congeners but usually BDE-28, −47, −99, −100, −153, −154, and −183 are analyzed. Among them, PCB-138 and -153 are the most abundant congeners [20]. Bodin *et al.* [17] found high levels of Σ PBDE in sea bass (11,004 pg/g dry weight-dw) from Antifer, France. Σ PBDEs ranged between 69 and 1100 pg/g (ww) in muscle of trout from Greenland and European high mountain lakes [21]. Despite the special attention to PBDE, in some cases a high amount of HBCDs was reported and sometimes were even higher than the PBDEs. Roosens *et al*. [22] found the sum of HBCDs to be at higher levels (4,500 ng/g, lw) than PBDEs (2,270 ng/g, lw) in most fis samples from the River Scheldt, Belgium. Eljarrat *et al*. [23] determined HBCD and PBDEs in barbel from the Cinca River, Spain. Highest HBCD and PBDE concentrations were reported as 750.4 and 297.9 ng/g (ww), respectively, depending on sampling tissues. Remberger *et al.* [24] determined high concentrations of HBCD in eel and pike samples (1,800 and 970 µg/kg, lw, respectively) in Sweden.

25.2.1.3 *Polychlorinated napthalenes (PCNs)*

PCNs are similar compounds to other chlorinated organohalogens, consisting of 75 congeners. Although the production of PCNs decreased in the late 1970s, they are still used in a number of countries [25]. Compared to the reports of PCBs and dioxins, studies on PCNs are limited. Parmanne *et al*. [26] reported the concentrations of PCNs in 90 herring collected from the Bothnian Sea, Northern Baltic. The total PCNs ranked between 40 and 430 ng/kg (ww). In other studies, the highest PCN concentrations were reported as 227 ng/kg (ww) in salmon [27], 39 pg/g (ww) in fis and shellfis samples among various foodstuffs from Spain [25], 360 ng/g (lw) in the muscle of pike from Sweden and the Baltic Sea [28], and 31,400 pg/g (ww) in walleye from the Detroit River, Canada, among fis species collected from Michigan waters [29].

Beside these dioxin-like compounds, limited studies are available in the literature regarding polycyclic aromatic hydrocarbons (PAHs) [30,31], polychlorinated terphenyls [32,33], per- and polyfluorinate alkyl substances [34], and polychlorinated alkanes [35] in aquatic organisms.

25.2.1.4 *Organochlorine pesticides (OCPs)*

The term "pesticide" has a very diverse range of meanings in terms of biological activity but it also encompasses many chemicals, exhibiting extremely diverse physical and

chemical properties [36]. The widespread use of pesticides has resulted in the presence of their residues in the aquatic environment. Once released into the environment, they are transformed into a range of different products due to their susceptibility to biotic and abiotic degradation. These compounds can be more mobile, more persistent, and occasionally more toxic to non-target organisms than parental pesticides [37]. Among OCPs; o,p′- and p,p′-isomers of 1,1,1-tichloro-2,2,bis (p-chlorophenyl) ethane (DDT), 1,1-dichloro-2,2-bis (p-chlorophenyl)ethylene (DDE) and (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) (DDD), hexachlorobenzene (HCB), α-,β-,γ- isomers of hexachlorocyclohexane (HCHs), chlordanes (CHLs; oxychlordane, trans- and cis-nonachlor, trans and cis-chlordane, and heptachlor epoxide), drins (aldrin, dieldrin, and endrin) are detected in seafood (Table 25.1).

Naso *et al*. [16] measured the HCBs and DDTs in marine species collected from the Gulf of Naples, Italy, including blue mussel, octopus, sea bass, mackerel, cuttlefish mullet, anchovy, and European hake. The highest HCB and DDTs values were observed in musky octopus (70 ng/g, lw) and in sea bass (971.3 ng/g, lw), respectively. In another study by Li *et al*. [38], who measured the OCPs in blood plasma of pelagic and benthic fis species from the Detroit River, Canada. Among pelagic fis species, the levels of ΣDDTs, ΣCHLs, and ΣHCHs ranged from 5.05 to 11.95, from 1.74 to 4.83, and from 0.07 to 0.84 ng/g (ww), respectively. The highest values were detected in northern pike, largemouth bass, and white bass, respectively. Among benthic species, OCP concentrations were even higher. Total DDTs and CHLs were highest in channel catfish whereas HCHs were highest in brown bullhead.

25.2.2 Heavy metals

Heavy metals (e.g. As, Cd, Hg, and Pb), especially at higher concentrations, threaten human health owing to their high toxicity, persistence, and tendency to accumulate in marine organisms, water, and sediments [39]. Metals enter the aquatic environment by atmospheric deposition, erosion of the geological matrix, or from anthropogenic sources, such as industrial effluent and mining wastes [40]. It is important to monitor trace element contents in seafood and much attention has been focused on potential human exposures to mercury (Hg). It exists in a number of inorganic and organic forms in water. Once released into the environment, inorganic Hg is converted to organic Hg (methylmercury, MeHg). MeHg is the most toxic chemical form, stable, and easily absorbed from the diet [41]. It is the most common form in seafood and could make up more than 90% of the total Hg [40]. MeHg concentrations in fis and shellfis are approximately 1,000 to 10,000 times greater than in other foods, including cereals, potatoes, vegetables, fruits, meats, poultry, eggs, and milk, among others [42].

Has Schön *et al*. [39] determined the heavy metal concentrations in different organs of fi e fis samples (carp, mullet, eel, tench, and sval) collected from the River Nevetra, Croatia. Hg and As levels were found to be the highest in the muscle of mullet (0.198 and 0.309 mg/kg, respectively), though Pb and Cd levels were the highest in kidney of carp (0.404 and 0.504 mg/kg, respectively). Fabris *et al*. [43] analyzed the metal concentrations in edible tissues of fis samples from Australian coasts. The highest As, Cd, and Hg levels were found in lobster (50.7 μg/g), abalone (0.12 μg/g), and snapper (0.17 μg/g), respectively. In another study, the highest Cd (0.07 mg/kg), Pb (0.035 mg/kg), and Hg (0.59 mg/kg) concentrations were reported for luvar among various fis species from Spanish markets [40]. The average level for Hg exceeded the maximum level of that specifie in European legislations. Only

Table 25.1 Concentrations of organochlorine pesticides (OCPs) in aquatic organisms

Location	Species	Year	HCH	CHL	HCB	DDT	DDE	DDD	Drins	ΣDDT	Unit	Reference
Turkey, Sir Dam Lake	Fish	2003	0.08–0.43	0.05–0.35	0.07–0.19	nd-4.3	4–901	0.35–54.3	–	14.4–77.4	ng/g ww	[79]
Spain, Balearic Islands	Invertebrates	1996–2005	0.2–0.8	–	0.1–0.2	–	–	–	–	1.9–5.2	ng/g ww	[80]
China, Pearl River Estuary-Daya Bay	Fish	2003–2004	0.20–0.26	0.84–4.78	–	–	–	–	–	40–80	ng/g ww	[81]
	Crab	–	0.16	0.53	–	–	–	–	–	57	ng/g ww	[81]
	Shrimp	–	0.07	0.24	–	–	–	–	–	10	ng/g ww	[81]
California, Salton lake	Tilapia	2000–2001	M: 0.3 L: 0.9	–	M: 2.6 L: 17.8	M: 3.9 L: 17.9	M: 9.6 L: 53	M: 1.5 L: 6.6	M: 2.9 L: 16.8	M: 15 L: 77.4	ng/g ww	[82]
	Corvine	–	M: 0.1 L: 1	–	M: 2 L: 10:2	M: 4.3 L: 35	M: 8.7 L: 76.1	M: 4.7 L: 17.7	M: 3.5 L: 45.2	M: 17.6 L: 128.8	ng/g ww	[82]
Greenland	Shorthorn sculpin	1994–1995	10	–	5.3	4.2	10	0.84	–	16	μg/kg ww	[83]
	Polar cod	–	33	–	11	14	19	11	–	44	μg/kg ww	[83]
	Blue mussel	–	0.60	–	0.068	0.12	0.31	0.060	–	0.49	μg/kg ww	[83]
Norway, Barents Sea,	Crustaceans	1995	29.7	15.2	16.5	–	–	–	–	18	ng/g lw	[84]
	Cod	–	41	100	65	–	–	–	–	42	ng/g lw	[84]
	Polar cod	–	37	76	39	–	–	–	–	17	ng/g lw	[84]
Russia, White Sea	Fish	1998–2000	0.26–3.40	0.48–4.30	0.1–2.36	–	–	–	–	5.1–21.3	ng/g lw	[85]
	Invertebrates	–	0.12–1.97	0.56–2.90	0.22–0.51	–	–	–	–	3.81–12.6	ng/g lw	[85]
China, Hong Kong markets	Freshwater fish	2004	–	–	–	nd-3.5	0.68–113	0.31–19.7	–	1.10–127	ng/g ww	[30]
	Marine fish	–	–	–	–	nd-133	1.78–244	nd–78.7	–	2.30–1018	ng/g ww	[30]

Abbreviations: HCH, hexachlorocyclohexan; CHL, chlordane; HCB, hexachlorobenzene; DDT, 1,1,1-tichloro-2,2,bis (p-chlorophenyl) ethane; DDE, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethylene; DDD, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane; Drins, aldrin, dieldrin, and endrin; L, liver; M, muscle; nd, not detected; ww, wet weight; lw, lipid weight.

one sample of luvar (0.18 mg/kg) exceeded the maximum levels of Cd in muscle meat [40]. Other studies reported in the literature are given in Table 25.2.

25.3 Aquaculture practices as a source of persistent contaminants

Aquaculture is the farming of aquatic organisms, including fish molluscs, and crustaceans by individuals, groups, or corporations, in order to enhance production. Products from aquaculture are generally regarded as safe and nutritious foods, but sometimes they show a higher risk of contamination by PEPs than that of products from freshwater or marine ecosystems [44]. The concentrations of contaminants may vary depending on the chemical properties of the compound, fis species, physiological state, type of farming, location of the farm, farming technology, management practices, and season [45].

It is usually reported that farmed species contain higher amounts of OCs and heavy metals than wild caught species. Carubelli *et al.* [46] found twice more PCB concentration in farmed sea bass than its wild counterpart due to its higher amount of fat. The concentrations tended to rise with age and there was no significan effect of different sampling farms. Similarly, Antunes and Gil [47] determined the PCB and total DDT values in muscles of farmed sea bass collected from two farms as 31 and 31.5 ng/g (dw), respectively, which was higher than in muscles of wild sea bass, 13 and 5.4 ng/g (dw), respectively. Higher levels of PBDE, PCB, and pesticides were detected in farmed salmon samples [31,48,49]. But Easton *et al.* [31] reported higher HCB and endrin levels in wild salmon. On the contrary, Zennegg *et al.* [50] determined the PBDE concentrations higher in wild whitefis samples from Swiss lakes (1.6–7.4 ng/g, ww) than farmed rainbow trout (0.74–1.3 ng/g, ww) from Swiss fis farms. Remberger *et al.* [24] reported higher HBCD concentrations in wild salmon (51 µg/kg, ww) than that of farmed salmon (6.7 µg/kg, ww) caught from the Baltic Sea, Sweden. In terms of heavy metals, higher Pb concentration was observed in wild eel compared to that of cultured eel [51]. Higher total Hg concentration was observed in wild rainbow trout (45 µg/kg), Atlantic salmon (56 µg/kg) [52], and in Pacifi Coast wild salmon (49.5 ng/g) [31]. Some studies have shown that commercial feed is the major contributor for PEP accumulation in cultivated species. PBDEs [31,49], PCBs [45,46], OCPs [31,47], PAHs [31], and heavy metals [53] were reported in feeds, and similar congener profile between feed and analyzed samples were obtained.

Beside the persistent contaminants, excessive and unconscious use of antibiotics in fis feed may have a negative impact on human health and on other aquatic organisms [54]. Antibiotics can be leached from the unconsumed feed and diffuse into the sediment at the bottom of the raising pens. They can be washed by currents to distant sites or remain in the sediments, altering the composition of the sediment microflor and select for antibiotic resistant bacteria [54,55]. The treatment of human infections becomes difficult either by the direct transfer of resistant pathogenic bacteria to humans or indirectly by the transfer of resistance genes from bacteria to human pathogens [56]. Many studies demonstrated the resistance to an antibiotic or multiple antibiotics in bacteria such as *Salmonella, Vibrio, Alteromonas, Enterococcus*, and *Pseudomonas* that were isolated from fish feed, and aquatic environments [56–58]. Moreover, heavy use of antibiotics can lead to elevated antibiotic residues in natural aquatic environments, aquaculture products, and wild fis [55,56]. Several antibiotics (tetracyclines and oxolinic acid) are known to be persistent, remaining in the sediments for months, and bioaccumulate [54].

Table 25.2 Concentrations of heavy metals in aquatic organisms

Location	Species	Year	Hg	Pb	As	Cd	Unit	Reference
Spain, River Turia	Eel, trout, & barbel	2000	–	0.0273–0.1018	0.0182–0.2279	0.0014–0.0049	µg/g ww	[86]
China, Zhejiang	Fish	1998	0.009–0.019	0.020–0.092	<DL-3	0.009–0.065	µg/g ww	[87]
	Cephalopod		0.016–0.024	0.027–0.041	3.8–7.2	0.16–0.86	µg/g ww	[87]
	Shellfish		0.012–0.027	0.055–0.332	1.1–1.5	0.119–7.19	µg/g ww	[87]
	Shrimp		0.01–0.015	0.01–0.141	2.7–6	0.146–0.606	µg/g ww	[87]
Gulf of Oman	Muscle of fish	2000–2001	0.343–2.35	0.005–0.551	0.834–10	<0.001–0.014	µg/g dw	[88]
	Liver of fish		0.287–4.65	<0.001–0.426	1.5–22.4	0.186–195	µg/g dw	[88]
	Bivalves		0.0087–0.315	0.098–3.92	11.1–156	1.17–21.9	µg/g dw	[88]
Italy, Adriatic Sea	Fish	2006	0.07–1.56	0.01–1.18	–	0.01–0.06	µg/g ww	[89]
	Cephalopod molluscs		0.1–0.55	0.04–0.17	–	0.18–0.59	µg/g ww	[89]
	Crustaceans		0.27–0.33	0.01–0.03	–	0.02–0.04	µg/g ww	[89]
Turkey, Marmara Sea	Fish	2005	0.0074–1.750	0.019–0.822	–	0.008–1.122	mg/kg	[90]
Spain, Galicia	Commercial fish% shellfish	2002–2003	0.17–0.97	0.1–0.75	–	0.1–1.11	mg/kg ww	[41]
Croatia, Adriatic Sea	Fish & shellfish		0.134–0.373	0.007–0.15	0.56–23.30	0.002–0.142	mg/kg ww	[61]
Trinidad and Venezuela	Green mussel		0.02–0.17	–	–	<0.01–0.61	µg/g ww	[91]
	Oyster		0.01–0.08	–	–	<0.01–0.53	µg/g ww	[91]
Spain, Local Markets	Fish		0.03–0.59	0.03–0.05	–	0.004–0.07	mg/kg ww	[40]
Turkey, Mediterranean Sea	Muscle of fish			2.98–6.12	–	0.37–0.79	µg/g dw	[60]
New Jersey, Local Markets	Commercial fish		0.01–0.65	0.04–0.34	0.2–3.3	0.00013–0.03	mg/kg ww	[59]

Abbreviations: L, liver; M, muscle; nd, not detected; DL, detection limit; ww, wet weight; dw, dry weight.

25.4 Factors affecting the occurrence of PEPs in seafood

In numerous studies, PEPs are detected in many freshwater (largemouth bass, carp, pike, trout, whitefish and salmon, etc.) and marine species (perch, tilapia, mussel, crab, and lamprey, etc.). They are usually found at higher levels in samples from industrialized, polluted sampling sites, and the Northern hemisphere [18]. Also coasts, ports, and estuaries are generally considered to be more heavily polluted than open waters. Qualitative and quantitative changes occur in bioavailability and bioaccumulation depending on several biotic (habitat, relative position within the food chain, detoxificatio mechanisms, sexual maturity/physiological factors, gender, lipid content, feeding behaviour, tissue composition, and metabolic capacity) and abiotic (physical and chemical properties of the chemicals, environmental characteristics such as temperature, pH, salinity, dissolved oxygen concentration, light, surface area, and surface type) factors [19,39,59]. None of these factors explain the differences in contaminants alone, as there is not a certain correlation between the contamination levels and hence in some cases contrary results are obtained. Organisms can have detoxificatio mechanisms and higher metabolic activity to metabolize these compounds, which can result in low tissue concentrations of PEPs. It is known that young individuals [60] and benthic organisms have higher metabolic activities [19].

Generally, organisms that have higher total lipid content, accumulate higher amounts of PEPs [18]. Once PEPs are ingested, they are distributed to different tissues in organisms, depending on the lipid content. The liver and gonads are the most contaminated, whereas muscle is generally less contaminated [17,21,61]. However, muscle is commonly analyzed because it is the main fis part consumed by humans.

The position within the food chain and feeding behaviour is another important factor affecting PEP concentrations in seafood. The species at higher trophic level have higher PEPs. It has been reported that benthic species display higher concentrations of PEPs than pelagic species due to their close contact with sediment particles [62]. The major biomagnificatio step in the food chain occurs from benthic species to fis and from fis to marine mammals [3].

Usually, a direct correlation exists between contaminant levels in tissues and age, thus increased body length/weight is reported [40]. However, there is a counterbalancing effect of dilution due to the increasing body weight and/or lowered metabolic activity with age [60]. There is not a direct relationship between gender and contamination levels, thus varying results are obtained within species. Lower concentrations of PEPs can be observed in females during spawning, due to the excretion of some of these compounds [21].

In addition to these factors, to compare results among organisms or habitats is rather complicated due to the great variety of congeners that have been analyzed, different expression of the results (such as ww, dw, and lw), different parts of samples that are used in analysis (muscle, liver, and gonad, etc.) and it is hard to specify a definit organism or habitat that is highly contaminated, unless it exceeds the certain limits.

25.5 Risk assessment and regulations

PEPs have adverse health effects. OCs act as immunosuppressive agents and endocrine disrupters. They affect cognitive functions, neurodevelopment, cause neurobehavioral deficits and neurotoxicity. Their exposure has also been shown to be associated with an increased

risk of diabetes, cancer, and cardiovascular diseases [13,63]. On the other, hand heavy metals have immunological, neurological, developmental, reproductive, genotoxic, carcinogenic, and systemic effects [64].

The consumers and food agencies are particularly concerned about the potential health effects of contaminated seafood. In order to investigate the intake of seafood originated contaminants on human health, the average daily exposure is calculated, but the results differ in countries due to the dietary habits of population, gender, and age [25,65]. To estimate the amount of certain contaminants that can be ingested over a lifetime without appreciable health risk, several international agencies have set provisional tolerable weekly intakes (PTWIs) or tolerable daily intakes (TDI). JECFA [66] established PTWIs for Cd, Pb, and Hg as 7, 25, and 1.6 μg/kg body weight (bw), respectively. TDI limits for DLCs are given as 1 to 4 pg TEQ/kg bw/day by WHO [67], 2 pg TEQ/kg bw/day by CoT [68] and ECSCF [69], 1 pg TEQ/kg bw/day by ATSDR [64], and 2.3 pg TEQ/kg bw/day by JECFA [12].

Maximum levels have also been implemented to keep human exposure to contaminants within safe limits. In the muscle meat of fis and fisher products, the maximum permissible levels were maintained at 4 pg/g WHO-TEQ (ww) for PCDD/Fs and 8 pg/g WHO-TEQ (ww) for the sum of PCDD/Fs and DL-PCBs excluding eel [70]. In terms of heavy metals, the limits range from 0.05 to 1.0, from 0.3 to 1.5, and from 0.5 to 1.0 mg/kg (ww) for Cd, Pb, and Hg, respectively, depending on the type of the organism [70]. Risk based thresholds for other OCs such as PBDEs and PCNs have not been established by any regulatory and public health agency. EC has also undertaken regulations in feed contaminants [71] and environmental fiel to reduce dioxin release [72].

25.6 Policies to reduce exposure to PEPs

As PEPs are generated from waste incineration and combustion processes, methods and technologies are being developed regarding waste treatment [73,74]. It is also necessary to implement measures to control industrial waste discharge and to avoid dispersal of these persistent toxic contaminants into the environment. Removal of skin [75] and some cooking processes [76] reduce contaminant levels in fish However, the amount of contaminant reduction is highly variable among species, contaminants, and cooking conditions [76,77], and in some cases higher concentrations are reported in the skin-off salmons than their skin-on counterparts [78]. In terms of aquaculture practices, control of feed composition could reduce or eliminate risks to human health. The active monitoring of heavy metals in all fis feed should be routinely carried out to assure the safety of the public.

25.7 Conclusions

Seafood, especially fish is an important component of a nutritious diet as a source of proteins, minerals, vitamins, and essential polyunsaturated fatty acids (PUFA) that are crucial to human health. On the other hand, they are bioaccumulators of environmental contaminants. Therefore, the risk of consuming contaminated seafood must be weighted in view of the health benefits In order to reduce human exposure to these contaminants, environmental pollution policies, monitoring tools, and regulations are required.

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26 Oxidation and stability of food-grade fish oil: role of antioxidants

Weerasinghe M. Indrasena and Colin J. Barrow

26.1   Introduction

Oxidation of lipids containing unsaturated fatty acids is a common and complicated phenomenon. Volatile compounds generated during the oxidation of fis oil contribute to the unfavourable fl vours and odours of the oil and the food products containing them. Although the initial mechanism of the oxidation seems simple, the mechanism and product mix become much more complicated and unpredictable during its progress, depending upon factors including the nature of the substrate and its environment. Oxidation of unsaturated fatty acids such as oleic, linoleic, and α-linolenic, predominantly from vegetable oils, and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from fis or microbial oil, produce several types of fl vour volatiles that affect the sensory properties of these oils. Antioxidants are commonly used to retard the oxidation and improve the quality of food-grade oils. This chapter will discuss mechanisms of lipid oxidation and methods to control lipid oxidation, including the use of antioxidants.
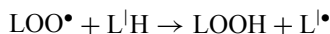
26.2   Process of oxidation

Oxidation of unsaturated fatty acids in oil may occur by two basic processes: autoxidation and photooxidation. Knowledge of the basic mechanisms of these two oxidation processes is required to understand the mechanism of the deterioration of the quality of food-grade fis oil, as well as how antioxidants can work in unsaturated oils or food products containing such oils.
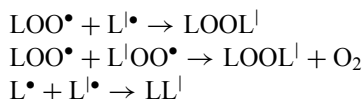
26.2.1   Autoxidation

Autoxidation occurs in three steps, known as initiation, propagation, and termination. These steps will be described in more detail below.

26.2.1.1 *Initiation*

Initiation:
$$LH \rightarrow L^\bullet$$
$$L^\bullet + O_2 \rightarrow LOO^\bullet$$

Propagation:
$$LOO^\bullet + L^|H \rightarrow LOOH + L^{|\bullet}$$

Termination:
$$LOO^\bullet + L^{|\bullet} \rightarrow LOOL^|$$
$$LOO^\bullet + L^|OO^\bullet \rightarrow LOOL^| + O_2$$
$$L^\bullet + L^{|\bullet} \rightarrow LL^|$$

Formation of free radicals ($L^\bullet$) from the unsaturated fatty acids (LH) is the beginning of the oxidative deterioration of the oil. These free radicals react with oxygen very rapidly to produce peroxyl radicals ($LOO^\bullet$), which are then converted to hydroperoxides, the primary products of autoxidation. Highly unsaturated fatty acids such as EPA and DHA are particularly susceptible to this reaction due to their large number of double bonds, and the high reactivity of the bridging methylene to radical initiation.

26.2.1.2 *Propagation*

During propagation, $LOO^\bullet$ produced in the initiation step can attack a double bond and abstract a hydrogen atom from another fatty acid ($L^|H$) forming a new free radical and hydroperoxide. The free radical continues to attack other unsaturated fatty acids in the system and propagate further in an exponential manner.

26.2.1.3 *Termination*

In the termination stage, free radicals begin to react with each other to form non-radical species ($L^\bullet + L^{|\bullet} \rightarrow LL^|$). Alcohols, acids, aldehydes, and ketones, as well as some other non-reactive substances, can be produced after the decomposition of hydroperoxides. Although the termination completes one cycle of lipid oxidation, this cycle continues to repeat by re-initiation (firs step) until all oxidizable substrate is depleted.

26.2.2 **Photooxidation**

Photooxidation or photosensitized oxidation occurs in a variety of systems, including food systems:

$$^|S + h\nu \rightarrow {}^1S^* \rightarrow {}^3S^*$$
$$^3S^* + {}^3O_2 \rightarrow {}^1O_2 + {}^|S$$
$$^1O_2 + LH \rightarrow LOOH$$
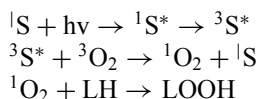
A photosensitizer in the oil or in a food product containing fis oil absorbs UV energy (hv) and becomes excited to move to a higher forbidden vibrational energy state ($^3S^*$). The

excited sensitizer can transfer its energy to produce singlet oxygen ($^1O_2$) from its ground state. This extremely reactive singlet oxygen species can attack unsaturated fatty acids due to the high electron density of the double bonds to produce peroxyl radicals (LOO$^\bullet$) and then hydroperoxides (LOOH):

$$^1O_2 + LH \rightarrow LOO^\bullet \rightarrow LOOH$$

The rate of photooxidation is much faster than autoxidation, since singlet oxygen reacts 1,000 to 1,500 times faster than triplet oxygen [1].

26.3   Factors affecting the rate of lipid oxidation

The nature and extent of oxidation depends upon the chemical structures of the fatty acids involved, the presence of minor constituents, and conditions of handling, processing, and storage. Physical factors such as surface area exposed to oxygen, oxygen pressure in the surrounding environment, temperature, and irradiation can contribute to the oxidation of fatty acids.

26.3.1   Oxygen

Oxygen is extremely important since it is highly reactive towards unsaturated fatty acids. When fis oil is exposed to air, oxygen can diffuse faster into the substrate if the pressure is high, even though the surface area is small, subsequently increasing the oxidation rate, when compared with systems that have larger exposing surface area and relatively low oxygen pressure. Therefore, the diffusion rate is more important than the surface area for diffusion controlled oxidation.

26.3.2   Physical form of oil

The rate of oxidation also depends on the nature of the physical form of the oil. The oxidation of bulk fis oil has an autocatalytic character, whereas the oxidation in the monomolecular layer occurs at a continuously decreasing rate [2]. Therefore, the mechanism and kinetics of autoxidation in bulk oil is different from that of monomolecular layers [3–5]. The viscosity of oil is also a factor, since penetration and mobility of oxygen in viscous oil is more difficul than in less viscous oils. Therefore, relatively high viscous triacylglycerols (TAG)-form of fis oil tend to be more resistant to oxidation than less viscous ethyl ester-form (EE) of fis oil.

26.3.3   Positional distribution of unsaturated fatty acids in the TAG molecule

TAG with unsaturated fatty acids in the Sn-2 position is more resistant to oxidation than those linked at the Sn-1 or Sn-3 positions, probably due to steric protection of the Sn-2 position. Also, there appears to be minimal influenc of the length of the carbon chain of saturated fatty acid on the oxidation of unsaturated fatty acids within the same molecule [6,7]. Because EPA and DHA are more stable at the Sn-2 positions, the preparation of fats with enrichment of EPA and DHA at the Sn-2 position should increase the storage stability of these oil.

26.3.4   Temperature

High temperature accelerates the oxidation of lipids, if appropriate initiators and oxygen are present. Therefore, thermal processing can significantl damage the sensory quality and stability of EPA and DHA containing oils.

26.3.5   Microcomponents in the oil

26.3.5.1   *Hydroperoxides*

There are several microcomponents that can influenc the rate of lipid oxidation. The hydroperoxides that are primary oxidation products are the initiators of the chain sequence causing the acceleration of the oxidation process in lipids.

26.3.5.2   *Free fatty acids*

The prooxidative effects of free fatty acids has been reported [8]. The carboxyl group of the free fatty acid can form a complex with hydroperoxide via hydrogen bonding, resulting in the decomposition of hydroperoxides into free radicals.

   Depending on the nature of the environment and substrate type, many other hydroxyl lipid compounds such as monoacylglycerols (MAG) and diacylglycerols (DAG) as well as sterols and fatty alcohols, which are components of natural lipids, can influenc the rate of lipid oxidation [9–11]. Some have reported that MAG and DAG have no effect on the oxidation, whereas others have seen a pronounced prooxidative effect [2]. It is likely that the variability in the observed effect depends on many other sub-factors, such as the reaction environment, molecular structure of the substrate, the presence of other minor components, as well as the physical and chemical nature of the medium.

26.3.5.3   *Thermally oxidized lipid compounds*

Some thermally oxidized compounds have shown prooxidant effects on refine and purifie soybean oil [12]. Similar or worse prooxidant effects can be expected from thermally processed fis oil, which contains highly unsaturated fatty acids.

26.3.5.4   *Heavy metals*

Mainly transient-valency metal ions catalyze the decomposition of hydroperoxides producing free radicals, which initiate further reaction chain. Redox active metals such as copper and iron are particularly problematic for promoting oil oxidation, even at trace levels. These metal ions (M) can be present in water and also leach into oil from containers:

$$LOOH + M^{n+} \rightarrow LO^{\bullet} + {}^-OH + M^{(n+1)+}$$
$$LOOH + M^{(n+1)+} \rightarrow LOO^{\bullet} + H^+ + M^{n+}$$
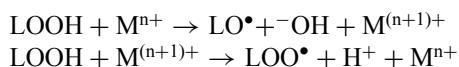
26.3.5.5   *Pigments*

Colourful natural compounds such as β-carotene, astaxanthin, canthaxanthin, and chlorophyll are present in some fis and microbial oils. Chlorophyll can contaminate fis oil during the preliminary extraction of oil from planktivorous fis such as anchovies, sardines, and

mackerels, especially in the industrial process of cooking and extracting in water. Plant pigments such as chlorophyll and pheophytins can act as photosensitizers, significantl increasing the initiation of light-induced lipid photooxidation. In contrast, these pigments may act as antioxidants in the dark [13]. Carotenoid pigments may also be anti- or pro-oxidants, although they are more often considered as antioxidants in oil [14–17].

26.3.5.6 *Non lipid components in food*

Oil must be completely free from moisture, since water can hydrolyze the TAG in the oil and initiate rancidity, which subsequently leads to rapid oxidation. The instability of free fatty acids contributes to this potentially rapid oxidation in the presence of water.

26.4   Food-grade fish oil

Consumption of fis oil in the form of capsules or liquid has been increasing due to multiple health benefit of the polyunsaturated fatty acids (PUFA) mainly EPA and DHA. However, oxidation of EPA and DHA leads to various fl vour volatile aldehydes and ketones that are formed as a result of the breakdown of hydroperoxides generated by oxidation. The composition and the intensity of these volatiles seem to vary, depending upon the substrate environment. Trans-2-hexenal and cis-4-heptenal have been identifie as possible oxidation markers of milk enriched with fis oil during storage [18], whereas hexenal, 2-nonenal, and 2,4-decadienals have been selected as quality indicators to monitor oxidation during the storage of microencapsulated fis oil [19]. The compounds1-penten-3one and 2,4-trans-trans-heptadienal could be used as volatile indicators for the oxidative status of herring oil [20]. Some volatile products generated during oxidation of fis oil are given in Table 26.1 [21], whereas some of the more pungent compounds are shown in Table 26.2 [22]. Unacceptable fis y fl vour in EPA and DHA containing oils can occur with the presence of sub ppm levels of a few volatiles, in particular 2,6-nonadienal, 4-heptenal, and 3,6-nonadienal. Dietary fis oils are encapsulated with antioxidants, not only to prevent oxidation and loss of EPA and DHA

Table 26.1   Different groups of some volatile oxidation products of fish oil. Adapted with permission from Kulas *et al*. [21]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA

Group	Volatile compound
Short-chain aldehydes	Acetaldehyde, propanal, 2-propenal, 2-butenal, butanal, pentenal, and 2-pentenal isomers
Long-chian aldehydes	Hexanal, 2,4-heptadienal isomers, nonanal, nonadienal, 2-decenal, and 2-undecenal
Diunsaturated aldehydes	2,4-Heptadienal isomers and nonadienals
Saturated aldehydes	Acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, and nonanal
Ketones	1-Penten-3-one
Alcohols	1-Penten-3-ol
Hydrocarbons	Pentane, 1-pentene, 2-methyl-1-butene, 1,3-pentadiene, 1-heptene, 3-methyl-1,4-heptadiene, octadiene, and octatriene
Acids	Formic acid, propanoic acid, butanoic acid, and hexanoic acid
Others	2-Ethylfuran

Table 26.2 Some volatile compounds that are responsible for odour and flavour of oxidized oils. Adapted from Karahadian & Lindsay [22]

Volatile compound	Odour	Taste
Hexanal	Grassy, greeny	Grassy
2-Hexenal	Greeny, fruity	Bitter
Heptanal	Gritty, oily, heavy	–
2-Heptenal	Creamy, oily, fatty, buttery	Bitter
2-Heptanone	Herby, pungent, fruity	–
Octanal	Citrus green	Plastic, painty
2,4-Heptadienal	Oily, fatty, hazelnut	Rancid, burnt
3-Octanone	Mushroom	–
2,6,-Nonadienal	Cucumber, green, melon	Fishy
3, 6-Nonadienal	Fatty	–
1-Octen-3-one	Earthy, musty	Creamy
1-Octen-3-ol	Metallic	Metallic

during storage, but also to avoid the formation of unacceptable taste profiles Bulk liquid oil can be combined with various fruit fl vours to mask unacceptable fl vours generated from oxidation. However, this masking only works at low levels of oxidation. Most of the volatile compounds that produce unacceptable fl vours in food-grade fis oils are readily removed by conventional steam deodorization. However, maintaining the oxidative stability is a more difficul problem, usually requiring a combination of antioxidants and appropriate storage conditions.

26.5 Control of lipid oxidation and improvement of the stability of fish oil
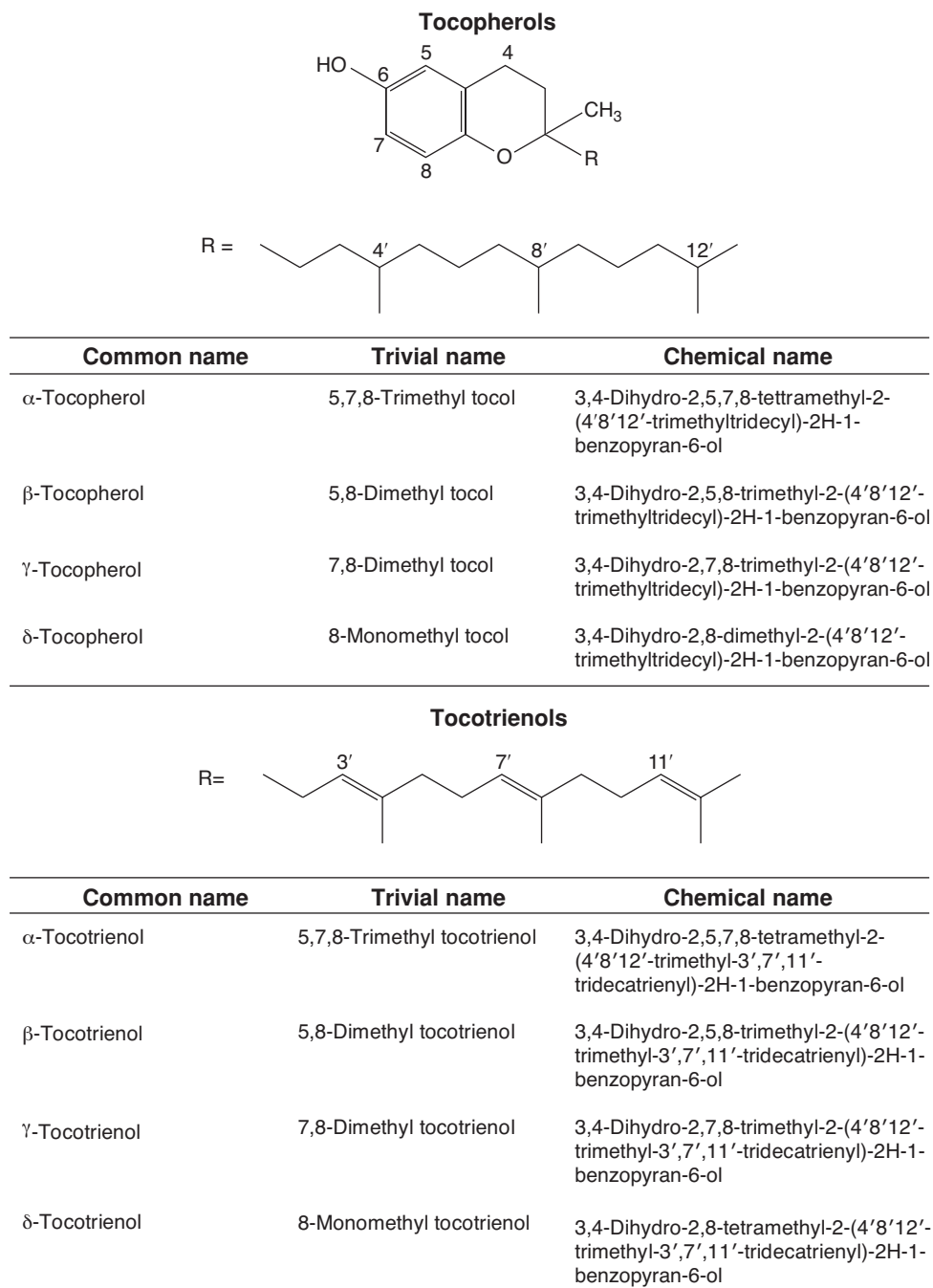
26.5.1 Careful handling and storage

Extreme care must be practised, especially during handling, processing, transferring and transporting, packaging, and storage of oil to minimize the oxidation through exposure to unfavourable conditions. High temperatures should be avoided in processing and the oil should not be exposed to oxygen and light at any time. Processed oil containing PUFA should be stored in the dark at or below $-20°C$ under an inert gas such as nitrogen or argon.

26.5.2 Inhibiting oxidation

Besides preventive measures, antioxidants and related compounds can be used to retard the oxidation of unsaturated fatty acids in fis and microbial oil. These compounds may have different inhibitory activities in the protection of oils against the process and propagation of oxidation.

26.5.2.1 Inhibiting photooxidation

Pigment or other highly conjugated or aromatic molecules can absorb light energy and become promoted to an "excited state" that initiates oxidation. Photosensitized oxidation

Tocopherols

Common name	Trivial name	Chemical name
α-Tocopherol	5,7,8-Trimethyl tocol	3,4-Dihydro-2,5,7,8-tettramethyl-2-(4'8'12'-trimethyltridecyl)-2H-1-benzopyran-6-ol
β-Tocopherol	5,8-Dimethyl tocol	3,4-Dihydro-2,5,8-trimethyl-2-(4'8'12'-trimethyltridecyl)-2H-1-benzopyran-6-ol
γ-Tocopherol	7,8-Dimethyl tocol	3,4-Dihydro-2,7,8-trimethyl-2-(4'8'12'-trimethyltridecyl)-2H-1-benzopyran-6-ol
δ-Tocopherol	8-Monomethyl tocol	3,4-Dihydro-2,8-dimethyl-2-(4'8'12'-trimethyltridecyl)-2H-1-benzopyran-6-ol

Tocotrienols

Common name	Trivial name	Chemical name
α-Tocotrienol	5,7,8-Trimethyl tocotrienol	3,4-Dihydro-2,5,7,8-tetramethyl-2-(4'8'12'-trimethyl-3',7',11'-tridecatrienyl)-2H-1-benzopyran-6-ol
β-Tocotrienol	5,8-Dimethyl tocotrienol	3,4-Dihydro-2,5,8-trimethyl-2-(4'8'12'-trimethyl-3',7',11'-tridecatrienyl)-2H-1-benzopyran-6-ol
γ-Tocotrienol	7,8-Dimethyl tocotrienol	3,4-Dihydro-2,7,8-trimethyl-2-(4'8'12'-trimethyl-3',7',11'-tridecatrienyl)-2H-1-benzopyran-6-ol
δ-Tocotrienol	8-Monomethyl tocotrienol	3,4-Dihydro-2,8-tetramethyl-2-(4'8'12'-trimethyl-3',7',11'-tridecatrienyl)-2H-1-benzopyran-6-ol

Fig. 26.1   Structures of tocopherols and tocotrienols.
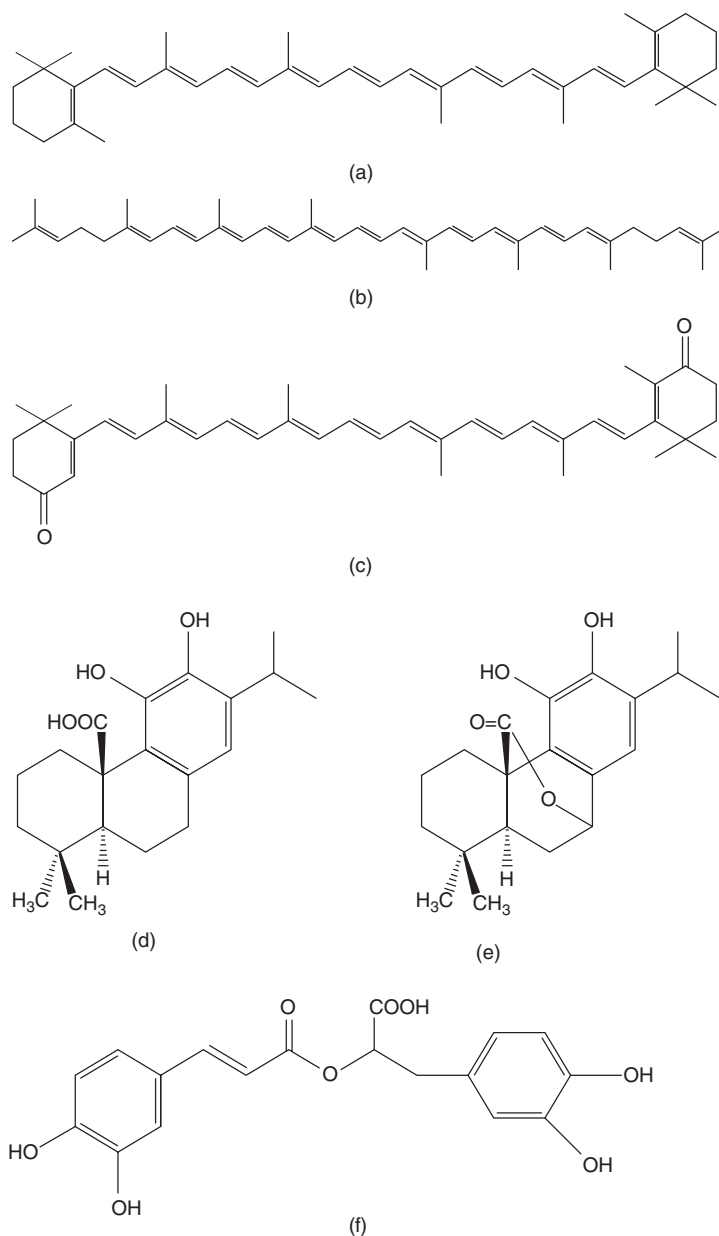
Fig. 26.2 Structures of some natural antioxidants: (a) β-Carotene; (b) Lycopene; (c) Canthaxanthin; (d) Carnosic acid; (e) Carnosol; and (f) Rosmarinic acid.

can be inhibited by adding simple quenching agents that quench molecular excitation. All unsaturated oils should be processed in a light controlled environment and the processed oil must be stored in the dark, light-preventive containers to avoid photooxidation. Some antioxidants, such as tocopherols (Fig. 26.1) and carotenoids (Fig. 26.2), that are excited-state quenchers, can also be used to inhibit photooxidation.

26.5.2.2 *Inhibiting autoxidation*

Many types of inhibitors such as antioxidants can be used to retard the free radical chain reaction process of autoxidation. Preventive antioxidants and chain-breaking antioxidants are the two main groups of compounds that can be used to inhibit autoxidation:

1) Preventive antioxidants include hydroperoxide decomposers, phospholipids, metal chelating agents, and singlet oxygen scavengers.
   - Hydroperoxide decomposers: these compounds inhibit lipid oxidation by inducing the decomposition of hydroperoxides, resulting in the stable alcohol or non-radical products.
   - Phospholipids: phospholipids can decompose hydroperoxides by a non-radical mechanism as well as by chelating traces of heavy metals that catalyze the oxidation process.
   - Metal chelating agents: these agents have shown a considerable effect on the oxidative stability of fis oil by scavenging prooxidant metal ions, resulting in limiting the production of chain inhibitors. Phosphoric, tartaric, citric, malic, and ascorbic acids (Fig. 26.3) possess good chelating effects. Citric acid is commonly used in industry for its dual purpose of being an antioxidative synergist and a chelator. Ethylenediaminetetraacetic acid (EDTA) is one of the best general chelating agents but cannot be used in fis oil due to poor solubility, unless the oil is in an emulsion or other aqueous system.
   - Singlet oxygen scavengers: carotenoids such as β-carotene, lycopene, canthaxanthin, lutin, and zeaxanthin can quench singlet oxygen ($^1O_2$), thereby limiting the oxidation. Carotenoids are naturally present in some algal oils rich in DHA (Fig. 26.2).
2) Chain-breaking antioxidants scavenge free radicals such as alkoxyl radicals ($RO^\bullet$) and peroxyl radicals ($ROO^\bullet$), resulting in the formation of antioxidant radicals. This antioxidant radical has a very low reactivity and therefore the propagation is effectively stopped:

$$ROO^\bullet + AH \rightarrow LOOH + A^\bullet$$
$$RO^\bullet + AH \rightarrow LOH + A^\bullet$$
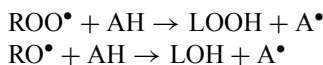
This type of antioxidants can produce a long lag period, which increases the induction period (IP) and this continues until about 90% of the antioxidant has been destroyed. However, the peroxidation continues at a very low rate at this phase and after 90% antioxidant is destroyed the oxidation becomes much faster, increasing to near the rate of unprotected oils. Types of common antioxidants and their effect on the storage stability of fis oil and other oil products containing unsaturated fatty acids are discussed in the following section.

26.6 Antioxidants

Antioxidants are the substances that retard the oxidation of a substrate significantl at low concentrations. They can be either natural or synthetic. Natural antioxidants are more popular than synthetic antioxidants for use with foods. The majority of natural antioxidants are phenolic compounds that include tocopherols and polyphenolic extracts from herbs. Tocopherols and tocotrienols are the most widely used natural antioxidants worldwide (Fig. 26.1). Other natural plant derived phenolic compounds such as rosmarinic, chlorogenic, vanillic, *p*-coumaric, *p*-hydroxybenzoic, caffeic, ferulic, sinapic, syringic, and 3,4-dihydroxybenzoic acid have antioxidant properties to different degrees. Spice herbs such as rosemary, oregano,
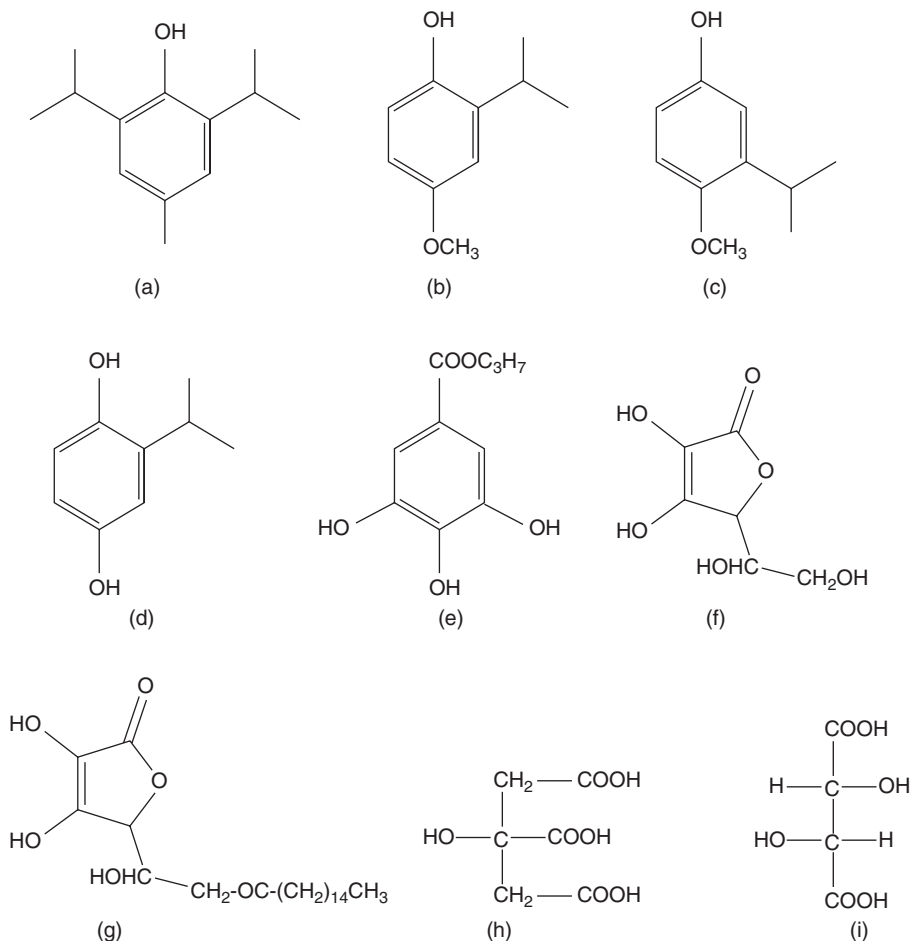
Fig. 26.3   Structures of some common synthetic antioxidants and chelating agents: (a) 2,6-di-tert-butyl-p-hydroxytoluene (BHT); (b) 4-methoxy-2-tert-butylphenol (2- BHA); (c) 4-methoxy-3-tert-butylphenol (3-BHA); (d) tert-butylhydroquinone (TBHQ); (e) Propyl gallate; (f) Ascorbic acid; (g) Ascorbyl palmitate; (h) Citric acid; and (i) Tartaric acid.

thyme, and cumin, as well as some rhizomes such as ginger and turmeric, have been used in fis culinary practices as antioxidants. Polyphenolic compounds such as rosmarinic acid, carnosic acid, and carnasol play a vital role as antioxidants in fis oil and many other food products containing unsaturated fat (Fig. 26.2).

Commonly used synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *t*-butylhydroquinone (TBHQ), and propyl gallate (Fig. 26.3). Although they are acceptable food-grade antioxidants with Food and Drug Administration (FDA) and have generally recognized as safe (GRAS) approvals, their use in foods including oil is limited due to customer concern and their "non-natural" status. Ascorbyl palmitate derived from the natural antioxidant ascorbic acid is used as a synergistic compound with tocopherol. However, both ascorbic acid and ascorbyl palmitate can be prooxidants

depending upon concentration and conditions, and so their oxidation status should be tested in the specifi application system in which they are to be used.

The difference in the antioxidant properties of antioxidants is a result of their chemical structures. The most important criteria in determining the antioxidant activity of a phenolic antioxidant is the position and the degree of hydroxylation of the aromatic ring [23–27]. Monophenols are less effective than polyphenols. The antioxidant activity of a monophenol can be increased by adding a second hydroxyl group to *ortho* and *para* positions and the antioxidant activity can be further increased by substituting the OH groups of phenol with methoxy groups. Substitutional groups such as esters normally reduce the antioxidant activity. For example, rosmarinic acid with two acid phenols is a more effective antioxidant than caffeic acid, since the antioxidant activity of the caffeic acid is reduced by the sugar moiety [2,23].

26.6.1 Mechanism of phenolic antioxidants

The mechanism of phenolic antioxidants starts with a lipid peroxy radical (LOO$^\bullet$) abstracting the hydrogen atom of the phenolic hydroxyl group. In the case of tocopherol, this results in the formation of a tocopheryl radical that reacts further to form α-tocopherol peroxide, α-toco quinine, and α-tocopherol quinine, thereby removing the lipid peroxy radicals from the medium. Two tocopheryl radicals can also be combined to form α-tocopherol dimmer [5].

The antioxidant effect of each tocopherol isomer depends on the hydrogen-donating power of the tocopherol to the lipid hydroperoxide. The hydrogen donating power of phenolic compounds also depends on the functional groups attached to the aromatic nucleus. Substituted groups at *ortho* or *para* positions should increase the electron density at the hydroxyl group and reduce the oxygen-hydrogen bond energy to facilitate the reactivity towards the lipid free radical. Therefore, substitution at the *meta* position may have only a limited antioxidant effect, *via* steric or stoicheometric effect [28].

The rate of production of non-radical products (A$^\bullet$ + LOO$^\bullet$ → AOOL, A$^\bullet$ + A$^\bullet$ → non radical) of sterically hindered phenols is greater than the rate of production of free radicals (A$^\bullet$ + LOOH → AH + LOO$^\bullet$, A$^\bullet$ + LH → AH + L$^\bullet$). The compounds with no steric hindrance favour the free radical forming reaction, whereas sterically hindered compounds favour the non-radical forming reaction. Therefore, sterically hindered phenols have a higher antioxidative effect than those without steric hindrance [2].

The antioxidant effect of individual tocopherol isomers depends on the concentration and hydrogen donating power of these isomers. Based on the structures of tocopherols and tocotrienols, the hydrogen donating power should be in the order of $\alpha > \beta > \gamma > \delta$. However, although the antioxidant activity *in vivo* is in the same order, the hydrogen donating power of tocopherol *in vitro* seems to be in the order of $\delta \geq \beta > \gamma > \alpha$.

Alpha-tocopherol is more prooxidative than γ-tocopherol in many systems. The prevention of oxidation of food-grade fis oils and fis oil containing food products is still a significan problem. The use of tocopherol antioxidants alone has not solved the sensory problems involved with fis oil oxidizing over the shelf-life of a functional food product. The usefulness of different tocopherols varies by food and food process. For example, γ-tocopherol has been found to be more effective than α-tocopherol in food products that involve high temperature heat treatment during the preparation [29].

26.6.2   Factors affecting the antioxidant activity of tocopherols

26.6.2.1   *Concentration and type of tocopherol*

The tocopherol type and concentration affect the formation of volatile secondary oxidation products as well as the composition of oxidation products in fis oil. High level of α-tocopherol directs the formation of more fl vour-potent aldehyde geometrical isomers as well as more fl vour-potent unsaturated aldehydes [21]. Addition of 100 ppm tocopherol can decrease the formation of hydroperoxides and C3-aldehydes in purifie fis oil TAG in the order of $\alpha > \gamma > \delta$, whereas the reverse order of the activity is true when the initial tocopherol level is 1,000 ppm [30]. The rate of the consumption of tocopherol is in the reverse order in both situations. Although tocopherols are the best-known and most widely used antioxidants, their prooxidant effect has been observed in lipid systems. The concentration of tocopherol is important, as these compounds can be either prooxidant or antioxidant, depending both on concentration and solution environment. Low levels of α-tocopherol are found to be more effective than higher levels, whereas high levels of γ-tocopherol are more effective than low levels [31,32]. This "inversion activity" of tocopherol homologues, which can occur as a result of concentration, depends on the reduction potential of the antioxidant [33,34]. Other tocopherol homologues can also be prooxidants to different degrees, the extent of prooxidant activity being dependent upon the solution environment, the tocopherol concentration, and the specifi oxidation-reduction potential of the tocopherol. However, the ratio of concentration of the substrate and antioxidant is an important factor in developing an optimized antioxidant for maximum stability of an oil. Initiation of oxidation generates an alkyl radical ($L^\bullet$) from an unsaturated fatty acid. The initiation reaction is very slow and hence is the rate determining step. However, this reaction can be catalyzed by heat, light, trace metals, and/or some specifi enzymes (e.g. lipoxygenases). Therefore, by controlling these factors, the initiation of oxidation can be delayed. Besides controlling these factors to prevent oxidation, the initiation step can be controlled by selective antioxidants. Tocopherols are particularly useful for inhibiting the initiation of oxidation, and controlling the rate of initiation, R_i:

$$\overset{R_i}{\underset{}{\downarrow}}$$
$$I + LH \rightarrow L^\bullet + IH$$
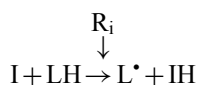
I = Initiator; R_i = Initial rate of reaction.

Gamma- and δ-tocopherols decrease R_i in methyl linoleate, independent of concentration. When a mixture of α-, γ-, and δ-tocopherol is added to EPA, as well as when α-tocopherol is added to methyl linoleate, R_i decreases to a certain level and then increases with increasing concentration, indicating the prooxidant activity of α-tocopherol. For example, 500 ppm α-tocopherol showed net prooxidant effect, 500 ppm γ-tocopherol showed no noticeable prooxidant effect, whereas δ-tocopherol showed antioxidant effect for the same substrate [35]. Low levels of α-tocopherol also seemed to have more antioxidant activity than the same amount of γ-tocopherol in rapeseed oil containing unsaturated fatty acids. The formation of hydroperoxides and relative consumption of α-tocopherol increases by increasing the amount of α-tocopherol from 50 μg/g to above 100 μg/g, whereas γ-tocopherol does not increase hydroperoxides at higher concentrations [36].

26.6.2.2   *Oxygen and temperature*

Factors such as surface area of oil exposed to oxygen, high oxygen pressure, heating, and irradiation can accelerate the initiation and propagation of the chain reaction of the oxidation process. These factors may even reduce the activity of antioxidant in the oil. Alpha-tocopherol is more effective at lower temperatures whereas γ-tocopherol is more effective at higher temperatures, although there is some substrate dependence. For example, α-tocopherol is more active than ferulic acid at 100°C in lard, but ferulic acid is more effective at room temperature [2].

   The temperature influence the antioxidant activity of tocopherols considerably. At lower temperatures antioxidant activity is in the order of $\alpha > \beta > \gamma > \delta$, whereas it is in the reverse order at higher temperatures [35]. The effect of high temperature on the anti- or prooxidant effect is related to the chemical composition of the medium. Gamma-tocopherol (0.01%) has a remarkable carry through antioxidant effect in crackers, pastry, and potato chips, etc. [29]. The antioxidant effect of α-tocopherol in these products seems to be lower than that of γ-tocopherol.

26.6.2.3   *Light*

Although tocopherols are known free radical scavengers, they can react with singlet oxygen in the presence of light and photosensitizers such as chlorophyll, as well as redox-active metal ions, thereby reducing their antioxidant effect. Thus, the quality of tocopherol affects its antioxidant activity in oil, and so these antioxidants must be stored properly prior to use to obtain the optimum activity.

26.6.2.4   *Substrate*

Methylene-interrupted fatty acids act as initiators of the lipid autoxidation process [37]. Therefore, the effect of antioxidant may be influence by the extent of unsaturation as well as whether the unsaturated fatty acid moiety is in the TAG form or EE form. Antioxidants are more effective on stabilizing TAG than EE. Also, fatty acid composition may be more important than the presence of antioxidant in the frying oil, whereas antioxidants may better stabilize the oil at lower temperatures. Oil with highly unsaturated fatty acids is more vulnerable to oxidation than oils with less unsaturated fatty acids. The presence of free fatty acids also decreases the oxidative stability of unsaturated oils. MAG, DAG, and fatty alcohols present as microcomponents have been shown to decrease the activity of antioxidant, since phenolic antioxidants can form complexes with hydroxyl group through hydrogen bonds [33,34]. The presence of metal ions in fis oil has a negative effect on the oxidative stability. This effect appears to be concentration dependent, with Fe being more prooxidant at high concentrations, while Cu can be more active at lower concentrations.

   Tocopherols are more effective in some animal fats such as lard than in vegetable oils, probably due to the lower unsaturated fatty acid content in lard [38]. Also, oils with higher levels of PUFA sometimes show the least tocopherol loss during frying. The reason for this may be due to the lack of any other prooxidants in the mixture, since α-tocopherol is more potent in highly oxidizable vegetable oils in the absence of prooxidants [39]. Although other compounds may increase the antioxidant potency of tocopherol, either by regeneration or chelation or both, the presence of some phenolic compounds may decrease the potency of

tocopherol at high concentrations (e.g. BHT + tocopherol). The presence of a prooxidant may cause the tocopherol to act as a prooxidant synergist. The viscosity of the substrate can also affect the efficien y of α-tocopherol and the presence of peroxyl radicals can decrease with increasing viscosity, partially due to poor mobility of oxygen and catalytic metal ions in highly viscous oils [40].

26.6.2.5 Polarity and pH of the medium

Polarity of the medium has a considerable effect on the activity of antioxidants. For example, prooxidant effects of α-tocopherol in linoleic acid in aqueous media are greater than those in other protic solvents and non-polar solvents [41], possibly due to high solubility of peroxyl radicals and other prooxidants in water. Low antioxidant effect of α-tocopherol in protic solvent systems may be due to the fact that H-bonding occurs between the phenolic group and the solvent, thereby reducing the antioxidant activity.

The effect of moisture depends on the type of antioxidant and the type of medium. Water can regenerate the tocopherol in water-soluble antioxidants and/or their synergists, increasing the stability of oil. Water can also have a protecting effect against the decomposition of hydroperoxides. However, a small amount of water in the medium also can enhance the metal catalysis by increasing the mobility of metal ions as well as facilitating the reduction of metals by the tocopherol. The pH of the polar medium can also have an effect, since lipid peroxides decompose at a much higher rate at high temperatures and low pH in the presence of prooxidants generating unfavourable fl vour volatiles.

26.6.2.6 Synergistic nature of tocopherols and other antioxidants

Tocopherols show their synergistic antioxidant activity in four different ways, depending upon other antioxidants present in the medium.

1) Increase antioxidant activity by the "regeneration of tocopherols" from its radical or oxidation products. For example, ascorbic acid and ascorbyl palmitate can regenerate α-tocopherol from its tocopheryl radical, thereby restoring its antioxidant activity [42–44]. Ascorbyl palmitate can protect tocopherols being consumed during the storage of fis oil. This synergistic antioxidant readily reduces the tocopheryl radical and regenerates tocopherol, subsequently increasing the effectiveness of tocopherol [45]. However, the second antioxidant may also promote prooxidation activity under certain conditions. For instance, ascorbic acid has a strong reducing activity for trace metal ions, thereby increasing the prooxidant effect. Therefore, trace metal ions must not be present if ascorbic acid is used as part of the antioxidant system. Synergistic phosphatidylethanolamine (PE) has the capability of regenerating tocopherol from its tocopheryl quinine [46], thereby increasing the antioxidant activity.
2) "Sparing effect" of tocopherols in the presence of other antioxidants that have the same or different antioxidant mechanisms. Phospholipids and some amino acids such as cysteine and methionine, which act as peroxyl radicals, also show a sparing effect to tocopherol. Alternatively, antioxidants having similar mechanisms can have a prooxidant effect equivalent to simply increasing the concentration of tocopherol. Although one reason for better antioxidant activity of a mixture of α-, γ-, and δ-tocopherols, than simply increasing the concentration of α-tocopherol alone, may be due to synergistic effect among different

tocopherols [47], the higher activity of a mixture is more likely be to due to better effect of γ- and δ-tocopherols than α-tocopherol.

3) Tocopherols can have a synergistic effect with another antioxidant through "metal chelation". For example, tocopherol is synergistic with phospholipids due to metal chelating properties of phospholipids [48]. A synergistic mixture of tocopherols, ascorbyl palmitate, and lecithin has been used to stabilize fis oil and microcapsules [49]. Although phenolic compounds in rosemary extracts have antioxidant properties [50], a mixture of rosemary extract, lecithin, tocopherol containing a low level of α- and high levels of γ- and δ-tocopherols, ascorbyl palmitate, and citric acid seems to have a great effect on the stabilization of bulk fis oil [51]. The antioxidant activity of this mixture can be further increased by using rosemary extract rich in carnosic and rosmarinic acid. However, the sensory quality of the stabilized oil should be monitored over a period of time, since some antioxidant components may induce off-fl vour formation in the oil during storage. Amino acids and some peptides show a similar synergistic effect with α-tocopherol. Citric acid also is synergistic with α-tocopherol when Cu ions are present [52]. The synergistic effect of citric acid is attributed to metal chelation. Melanoidins, which are the reaction products of Millard browning reaction, can also be synergistic with tocopherol [53].

4) "Physical rearrangement" of phospholipids with tocopherols can produce a synergistic antioxidant effect [54]. Phospholipids are good emulsifying agents in the oil. They form reverse micelles in the form of microemulsions, which solubilize tocopherols. In these emulsions, the active phenolic groups are positioned near the polar region where peroxyl radicals are formed, thereby facilitating the "scavenging activity" of these radicals by the active sites in the polar region.

26.7   Selection of an antioxidant

One of the most common methods for controlling lipid oxidation is the use of antioxidants. Natural antioxidants are becoming the choice of antioxidant in food and gaining global acceptance for their antioxidant performance and safety profil when compared with synthetic antioxidants. However, many natural antioxidants have not yet been tested for their carcinogenic and mutagenic effect, although almost all synthetic antioxidants have been tested.

Choosing the correct antioxidant combination for use in food-grade fis oil or food system containing fis oil is challenging. Primary selection criteria include the potency of the antioxidants in the system of interest. The efficien y of antioxidants can be pre-tested by determining one or few oxidative stability indices, such as the oxidative stability index (OSI) or induction time (IT), peroxide value (PV), *p*-anisidine value (*p*-AV), and by measuring the thiobarbituric acid reactive substances (TBARS) of unsaturated fatty acids after mixing the oil or oil product with antioxidants. Since most of these indices do not directly correlate with the sensory profil of oil, it is important to also monitor the sensory changes during processing and over the storage life of the product. Most importantly, the antioxidants themselves should not impart the sensory properties of the oil or food. The following criteria are helpful when selecting antioxidants. They should be safe as a food ingredient; not impart colour, odour, or fl vour; be effective at low concentrations; be active after oil or food processing; be stable in the finishe product during storage; be fat soluble for oil stability; be readily available at low cost; preferably be natural; fulfi regulatory and quality control requirements globally; and be free from allergens as well as short- and long-term side effects.

26.8   Conclusions

Fish and microbial oils containing EPA and DHA can rapidly oxidize in the presence of oxygen. Radical initiators present in oil as well as light can initiate oxidation and promote the rapid rancidity of these oils. When these oils are added to food, food processing steps can further damage the oil, as can storage. In foods, the oil is exposed to the complicated food matrix, which may contain radical initiators and catalysts such as Fe and Cu. Correct selection of antioxidants is critical to protect EPA and DHA containing oil or foods from oxidation. Combination of different tocopherols may be more effective than any single homologue due to synergism. Other antioxidants such as rosemary extract, ascorbyl palmitate, and phospholipids can also be synergistic with tocopherols. Antioxidant combinations should be tested for prooxidant activity, since this can vary depending upon the oil and food matrix. It is not possible to fully predict activity of antioxidant combinations in a complex matrix from firs principles. So empirical testing is required to obtain the optimum concentrations and ratios. The development of antioxidant systems for EPA and DHA containing oils and foods is an ongoing research area that will continue to advance as these fatty acids become more and more popular as healthy food ingredients.

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27 Global legislation for fish safety and quality

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27.1 Introduction

The European Union (EU) Food Safety Legislation aims to merge, harmonise, and simplify very detailed and complex hygiene requirements currently scattered over fi e directives. The overall aim is to create a single hygiene regime covering food and food operators in all sectors, together with effective instruments to manage food safety and any possible food crises throughout the food chain. Food producers will bear primary responsibility for the safety of food through the use of a "Hazard Analysis and Critical Control Points" (HACCP) System [1].

The US Constitution prescribes the responsibilities of the government's three branches: executive, legislative, and judicial, which all have roles that underpin the nation's food safety system. The US food safety system is based on strong, fl xible, science-based federal and state laws, and industry's legal responsibility to produce safe foods. Federal, state, and local authorities have complementary and interdependent food safety roles in regulating food and food processing facilities [2]. This chapter covers global legislation issues for fis safety and quality.

27.2 Global legislation in fish and fishery products

EU, US, Canadian, Australian, and Japanese legislation in fis and fisher products are discussed in detail.

27.2.1 EU legislation

The EU food safety policy encompasses the whole of the animal and human food chain. It provides extensive legislation and outlines the responsibility of producers and suppliers in helping to ensure safe quality of the food supply. The EU regulations are among the most stringent in the world [3].

In EU legislation, there are fi e regulations that refer to food safety issues and they cover many kinds of food, including fis and fisher products. In particular, Regulation

(EC) No 178/2002 [4] (entry into force 1 January 2005) lays down the general principles and requirements of food law, establishes the European Food Safety Authority, and sets out procedures in matters of food safety. Regulation (EC) No 852/2004 [5] (entry into force 1 January 2006) lays down general rules for food business operators on the hygiene of foodstuffs. Regulation (EC) No 853/2004 [6] (entry into force 1 June 2006) lays down specifi hygiene rules for food of animal origin. Regulation (EC) No 854/2004 [7] (entry into force 1 January 2006) lays down specifi rules for the organisation of officia controls, and finall , Regulation (EC) No 882/2004 [8] (entry into force 1 January 2006) lays down general rules for the performance of officia controls to verify compliance with rules aimed at preventing, eliminating, or reducing acceptable levels of risks to humans and animals.

According to Directive 79/923/EEC [9] Member States:

1. shall, within a two-year period following the notificatio of this Directive, designate shellfis waters;
2. may subsequently make additional designations;
3. may revise the designation of certain waters, owing in particular to factors unforeseen at the time of designation; and
4. shall establish programmes in order to reduce pollution and to ensure that designated waters conform to the values set by the Member States, within six years following designation.

Following Directive 91/67/EEC [10] (entry into force 1 January 1993), the placing on the market of aquaculture animals shall be subject to the following general requirements:

1. they must show no clinical signs of disease on the day of loading;
2. they must not be intended for destruction or slaughter under a scheme for the eradication of a disease; and
3. they must not come from a farm which is subject to a prohibition for animal health reasons and must not have been in contact with animals from such a farm.

Following Directive 91/492/EEC [11] (entry into force 14 October 1991), the placing on the market of live bivalve molluscs for immediate human consumption shall be subject to the following conditions:

1. they must originate from production areas, which comply with the requirements laid down in this Directive; in the case of Pectinidae, this provision shall apply only to aquaculture products;
2. they must have been harvested and transported from the production area to a dispatch centre, purificatio centre, relaying area, or processing plant under the conditions laid down in this Directive;
3. where provided for in this Directive, they must have been re-laid in suitable areas approved for that purpose;
4. they must have been handled hygienically, and where appropriate, they must have been purifie in establishments approved for that purpose;
5. they must comply with the criteria set out in this Directive;
6. health controls must have been carried out;
7. they must have been appropriately wrapped;

8. they must have been stored and transported under satisfactory conditions of hygiene; and
9. they must bear a health mark.

With regard to Directive 91/493/EEC [12] (entry into force 1 January 1993), the placing on the market of aquaculture products shall be subject to the following conditions:

1. they must have been slaughtered under appropriate conditions of hygiene; and
2. they must have been handled and, where appropriate, packaged, prepared, processed, frozen, defrosted or stored hygienically in establishments and they must have been stored and transported under satisfactory conditions of hygiene.

The placing on the market of the following products shall be forbidden:

1. poisonous fis of the following families: Tetraodontidae, Molidae, Diodontidae, Canthigasteridae; and
2. fisher products containing biotoxins such as ciguatera toxins or muscle-paralysing toxins.

The Directive 92/48/EEC [13] (entry into force 1 January 1993) makes it clear that the sections of vessels or the containers reserved for the storage of fisher products must not contain objects or products liable to transmit harmful properties or abnormal characteristics to the foodstuffs. These sections or containers must be well designed so as to allow them to be cleaned easily and to ensure that meltwater cannot remain in contact with the fisher products. The fisher products shall be handled and stored in such a way as to prevent bruising.

The purpose of Directive 2000/60/EC [14] (entry into force 22 December 2000) is to establish a framework for the protection of inland surface waters, transitional waters, coastal waters, and groundwater which:

1. prevents further deterioration and protects and enhances the status of aquatic ecosystems and, with regard to their water needs, terrestrial ecosystems and wetlands directly depending on the aquatic ecosystems;
2. promotes sustainable water use based on a long-term protection of available water resources;
3. aims at enhanced protection and improvement of the aquatic environment, *inter alia*, through specifi measures for the progressive reduction of discharges, emissions, and losses of priority substances and the cessation or phasing-out of discharges, emissions, and losses of the priority hazardous substances;
4. ensures the progressive reduction of pollution of groundwater and prevents its further pollution; and
5. contributes to mitigating the effects of flood and droughts.

Directive 2006/88/EC [15] (entry into force 14 December 2006) established:

1. animal health requirements for the placing on the market, importation, and transit of aquaculture animals, and their products;
2. minimum measures to prevent diseases in aquaculture animals; and
3. minimum measures to be taken in response to suspected or established cases of certain diseases in these animals.

Exotic diseases are those that are not established in Community aquaculture and whose pathogen is not present in Community waters. These include the following diseases: epizootic haemopoietic necrosis, infection with *Bonamia exitiosa*, infection with *Xenohaliotis californiensis*, *Taura syndrome*, or even yellow-head disease.

Decision 97/296/EC [16] (entry into force 1 July 1997), lists countries and territories from which importation of fisher products in any form intended for human consumption is authorised.

The Regulation (EC) No.104/2000 [17] (entry into force 2 February 2000) claims for the purposes of this regulation, that producer organisation means any legal entity:

1) set up on the own initiative of a group of producers of one or more of the products, in the case of frozen, treated, or processed products, as the operations in question have been carried out on-board fishin vessels;
2) established for the purpose of ensuring that fishin is carried out along rational lines and that conditions for the sale of the members' products are improved, by taking such measures as will encourage the planning of production, promote the concentration of supply, stabilise prices, encourage fishin methods; and
3) the rules of association which require its producer members, in particular, to apply to fishin production and marketing.

The EU legislation related to specifi provisions – fis and fisher products, are summarised in Table 27.1.

27.2.2 US legislation

In US legislation, there are four Acts that refer to food safety issues. These are Consumer Product Safety Act (1972), Food Quality Protection Act (1996), Food Safety Act (2002), and Public Health Security and Bioterrorism Preparedness and Response Act (2002).

According to procedures for the safe and sanitary processing and importing of fis and fisher products (1995) [18] "fish means fresh or saltwater finfis crustaceans, other forms of aquatic animal life (including, but not limited to, alligator, frog, aquatic turtle, jellyfish sea cucumbers, sea urchin, and the roe of such animals) other than birds or mammals, and all molluscs, where such animal life is intended for human consumption; "fisher products" means any human food product in which fis is a characterising ingredient. The HACCP plan shall, at a minimum, list the food safety hazards that are reasonably likely to occur and that thus must be controlled for each fis and fisher product. Food safety hazards are reasonably likely to occur as a result of:

a) natural toxins;
b) microbiological contamination;
c) chemical contamination;
d) pesticides;
e) drug residues;
f) decomposition in scombroid toxin;
g) parasites;
h) unapproved use of direct or indirect food or colour additives; and
i) physical hazards.

Table 27.1 EU legislation (main points and comments) focused on specific provisions: fish and fishery products

Directive	Title	Main points	Comments
Directive 79/923/EEC (entry into force 5/11/1981)	Quality required of shellfish waters	• Applicable to coastal and brackish waters in order to support shellfish life. Criteria for minimum demanded quality of shellfish waters.	→ Directive 79/923/EEC will be repealed in 21/12/2013 and only Directive 2000/60/EC will be gone.
Directive 91/67/EEC (entry into force 1/1/1993)	The animal health conditions governing the placing on the market of aquaculture animals and products	• The placing on the market of aquaculture animals shall be following general requirements. • Aquaculture animals and products must come from third countries or parts thereof appearing on a list.	→ There are four Directives and a Regulation that amended this Directive. The last one is Regulation (EC) No. 806/2003 (entry into force 5/6/2003).
Directive 91/492/EEC (entry into force 14/10/1991)	Laying down the health conditions for the production and the placing on the market of live bivalve molluscs	• This Directive applies to echinoderms, tunicates, and marine gastropods. • Provisions for Community production and imports of live bivalve molluscs from third countries.	→ Directives 97/61/EC (entry into force 18/11/1997) and 97/79/EC (entry into force 19/2/1998) amended this one.
Directive 91/493/EEC (entry into force 1/1/1993)	Laying down the health conditions for the production and the placing on the market of fishery products	• Fishery products which are to be marketed live must at all times be kept under the most suitable survival conditions. • Fishery products may not be handled except in factory ships or establishments conforming to the standards laid down in this Directive.	→ This Directive was amended by two Directives and one Regulation. The last one was Regulation (EC) No 806/2003 (entry into force 5/6/2003).
Directive 92/48/EEC (entry into force 1/1/1993)	Laying down the minimum hygiene rules applicable to fishery products caught on board certain vessels	• The general hygiene conditions shall apply to fishery products handled on board fishing vessels and the additional hygiene conditions shall apply to fishing vessels designed and equipped to preserve fishery products on board under satisfactory conditions for >24 hours.	

(Continued)

Table 27.1 (Continued)

Directive	Title	Main points	Comments
Directive 2000/60/EC (entry into force 22/12/2000)	Establishing a framework for Community action in the field of water policy	• EU provides for the management of inland surface waters, groundwater, transitional waters, and coastal waters in order to prevent and reduce pollution, promote sustainable water use, protect the aquatic environment, and improve the status of aquatic ecosystems.	→ Decision No 2455/2001/EC (entry into force 16/12/2001) and Directive 2008/32/EC (entry into force 21/3/2008) amended this Directive.
Directive 2006/88/EC (entry into force 14/12/2006)	Animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals	• The animals concerned are fish, molluscs, and crustaceans, and their products. • It is not including ornamental animals bred in an aquarium not intended for sale, wild animals introduced directly into the food chain, and animals intended for the production of fish meal, fish oils, and similar products.	
Decision 97/296/EC (entry into force 1/7/1997)	Drawing up the list of third countries from which the import of fishery products is authorised for human consumption	• List of countries and territories from which importation of fishery products in any form intended for human consumption. • Certification that fishery products exported to the Community.	→ There are 32 Decisions that amend Decision 97/296/EC from 1997 to 2005 and they complete the list of third countries from which the import of fishery products is authorised for human consumption. The last one was Decision 2005/501/EC.
Regulation (EC) No 104/2000 (entry into force 2/2/2000)	The common organisation of the markets in fishery and aquaculture products	• A common organisation of markets in fishery products is hereby established, comprising a price and trading system on competition.	→ Repealing Regulations (EEC) No.3759/92, No.105/76 and No.1772/82 from 1/1/2001. → There are three Regulations which amended this one. The last one was Regulation (EC) No 1759/2006 (entry into force 2/12/2006).

Processors of smoked or smoke-fl voured fisher products shall include in their HACCP plans how they are controlling the food safety hazard associated with the formation of toxin by *Clostridium botulinum*, for at least as long as the shelf-life of the product under normal and moderate abuse conditions.

For the purpose of Sustainable Fishery Act [19] "commercial fishing means fishin in which the fis harvested, either in whole or in part, are intended to enter commerce or enter commerce through sale, barter, or trade and "fishin community" means a community which is substantially dependent on or substantially engaged in the harvest or processing of fisher resources to meet social and economic needs, and includes fishin vessel owners, operators, crew, and US fis processors that are based in such a community. The North Pacifi Council and the Secretary shall establish a western Alaska community development quota programme under which a percentage of the total allowable catch of any Bering Sea fisher is allocated to the programme. To be eligible to participate in the western Alaska community development quota programme, a community shall:

1. be located within 50 nautical miles from the baseline from which the breadth of the territorial sea is measured along the Bering Sea coast from the Bering Strait to the westernmost of the Aleutian Islands, or on an island within the Bering Sea;
2. not be located on the Gulf of Alaska coast of the north Pacifi Ocean;
3. meet criteria developed by the Governor of Alaska, approved by the Secretary, and published in the Federal Register;
4. be certifie by the Secretary of the Interior pursuant to the Alaska Native Claims Settlement Act to be a Native village;
5. consist of residents who conduct more than one-half of their current commercial or subsistence fishin effort in the waters of the Bering Sea or waters surrounding the Aleutian Islands; and
6. not have previously developed harvesting or processing capability sufficien to support substantial participation in the ground-fis fisherie in the Bering Sea, unless the community can show that the benefit from an approved Community Development Plan would be the only way for the community to realise a return from previous investments.

A summary of US legislation focused on fis and fisher products is given in Table 27.2.

27.2.3 Canadian legislation

The Fish Inspection Act [20] applies to the shipment of fis or marine plants from one province to another, as though the shipment from a province were an export and the shipment

Table 27.2 US legislation (title and main points) related to fish and fishery products

Title	Year	Main points
Procedures for the safe and sanitary processing and importing of fish and fishery products	1995	• Definitions (fish, fishery products, etc.) • Current good manufacturing practice • Special requirements for imported products and for processing smoked, smoke-flavoured fishery products, fresh and frozen molluscan shellfish
Sustainable Fishery Act	1996	• Fishery monitoring, research and management plans • Fisheries financing and capacity reduction

into a province were an import. The Governor in Council may, for the purpose of regulating the export or import of fis and containers, make regulations:

1. prescribing grades, quality, and standards of fish
2. defining for the purposes of Section 10, the expressions "tainted", "decomposed", and "unwholesome";
3. respecting the processing, storing, grading, packaging, marking, transporting, and inspection of fish
4. respecting the quality and specification for containers and the marking and inspection of containers;
5. requiring the registration of establishments and the licensing of persons engaged as principals or agents in the export or import of fis or containers,
6. prescribing the requirements for the equipment and sanitary operation of establishments, of premises operated by an importer for the purpose of importing fish and of any boats, vehicles, or other equipment used in connection with an establishment or in connection with fishin or the import or export of fish
7. prescribing fees for registration of establishments, issue of licences, and grading and inspection services;
8. prohibiting the sale or offering for sale or holding in possession for sale of any fis or containers under any grade name or standard prescribed by regulations made under this Part, unless all the requirements of this Part and the regulations thereunder with respect thereto have been complied with, or under any name calculated to mislead or deceive;
9. prescribing the manner in which samples of any fis may be taken;
10. prohibiting or restricting any export or import of, or any attempt or offer to export or import, any fis or containers, unless all the requirements of this Part and the regulations thereunder with respect thereto have been complied with; and
11. establishing requirements governing the seizure and detention of fis and containers.

In agreement with the Freshwater Fish Marketing Act [21], the corporation is established for the purpose of marketing and trading in fish fis products, and fis by-products in and outside Canada and, in addition to the powers conferred by other provisions of this Act and by any other Act, has for that purpose power to:

1. buy fis and dress, fillet freeze, package, or otherwise prepare fis for market;
2. buy, manufacture, or produce fis products and fis by-products and package or otherwise prepare fis products and fis by-products for market;
3. store, ship, insure, import, export, market, sell, or otherwise dispose of fish fis products, and fis by-products bought, prepared, manufactured, or produced by it;
4. purchase, lease, or otherwise acquire and hold, sell, or otherwise deal with any real property;
5. establish branches or employ agents in Canada or elsewhere;
6. invest any money in its possession or under its control that in its opinion is not immediately required for the purposes of its operations, in securities of or guaranteed by the Government of Canada and sell any securities so acquired by it and re-invest the proceeds or any part of the proceeds thereof in like manner;
7. borrow money from any bank on the credit of the Corporation;

Table 27.3   Canadian legislation for fish and fishery products

Title	Year	Main points
Fish Inspection Act	1985	• Regulating the export or import of fish and containers • Regulating marine plants • This Act applies to the shipment of fish or marine plants
Freshwater Fish Marketing Act	1985	• Export trade in fish • Marketing and trading in fish, fish products, and fish by-products

8. make loans of working capital on a seasonal basis to persons engaged in fishin for commercial purposes in a participating province; and

9. do all such other things as are necessary or incidental to the exercise of any of its powers or the carrying out of any of its functions under this Act.

Some representative points and comments of the Acts regarding fis and fisher products are given in Table 27.3.

27.2.4 Australian legislation

In the Fisheries Act [22], a person must not unlawfully:

1. bring noxious fisherie resources, or cause noxious fisherie resources to be brought, into Queensland;

2. possess, rear, sell, or buy noxious fisherie resources; or

3. release noxious fisherie resources, or cause noxious fisherie resources to be placed or released, into Queensland waters. Maximum penalty is 2,000 penalty units. A person must not unlawfully:

   i) bring non-indigenous fisherie resources, or cause non-indigenous fisherie resources to be brought, into Queensland;

   ii) possess, rear, sell, or buy non-indigenous fisherie resources;

   iii) release non-indigenous fisherie resources, or cause non-indigenous fisherie resources to be placed or released, into Queensland waters; or

   iv) release non-indigenous fisherie resources, or cause non-indigenous fisherie resources to be placed or released, in a waterway or lake in a wild river area.

Maximum penalty is 2,000 penalty units. The chief executive may declare an area to be a quarantine area because of the presence, or suspected presence, of a declared disease in the area (a declared quarantine area). The quarantine declaration may make provision about the matters the chief executive considers necessary or desirable for the management, control, and elimination of the declared disease.

According to the Fisheries Management Act [23], the regulations may declare that fis of a specifie species that do not comply with a minimum size, maximum size, or range of sizes specifie for fis if that species are prohibited size fish The regulations may prescribe the method of determining the size of any class of fish In this section, "size" means measurement

Table 27.4 Australian legislation for fish and fishery products

Title	Year	Main points	Comments
Fisheries Act	1994	• Assessment of development applications for construction of raising of waterway barrier work • Particular fisheries development also requires a resource allocation authority • Noxious and nonindigenous fisheries resources and aquaculture fish	→ Last amendment of this Act was in 2008
Fisheries Management Act	1994	• Fishery management strategies • Commercial share management fisheries • Licensing and other commercial fisheries management	→ This Act was amended in 2007

or weight, or measurement and weight. In this Act, "fish includes:

1. oysters and other aquatic molluscs;
2. crustaceans;
3. echinoderms; and
4. beachworms and other aquatic polychaetes.

In this Act, "fish also includes any part of a fish However, in this Act, "fish does not include whales, mammals, reptiles, birds, amphibians, or other things excluded from the definitio by the regulations. For the purposes of this Act, a fisher may be identifie by reference to any one or more of the following:

1. species or other class of fish
2. an area of waters or seabed;
3. a method of fishing
4. class of boat;
5. class of persons; and
6. purpose of activities.

The main points of these Acts are given in Table 27.4.

27.2.5 Japanese legislation

According to bacteriological and chemical guidelines for fis and seafood in Japan, microbial count should be more than 100,000/g product. For oyster, the microbial count should be less than 50,000/g product. Only edible parts of globe fis can be consumed, and types from sea areas where fishin is permitted. Shellfis could cause paralytic or diarrhoeal shellfis poison. Food shall not include unauthorised antibiotic residues. Fish and shellfis shall not include residues on synthetic antimicrobials. Food may contain residues of these drugs only when the drugs comply with standards established by the law. The drugs which are used should be licensed [24].

Food sanitation law refers to fish-past products, which include fis ham, fis sausage, whale bacon, and other similar products. This law shall be applied to all fish/shellfi selling businesses, fish/shellfi auctioning businesses, fish-past product manufacturing

businesses, including businesses which manufacture fis ham, fis sausage, whale bacon, and other similar products. For a meat product, whale meat product, fis sausage, fis ham, or specially wrapped kamaboko (fish-past cake), which was tightly packed into a hermetic container/package and pasteurised either by holding its centre at 120°C for 4 minutes or by any other method providing a comparable or superior effect, the method of pasteurisation. For fis sausage, fis ham, or a specially wrapped kamaboko, whose pH is not more than 4.6 or whose water activity is not more than 0.94 (excluding canned or bottled products), the pH or water activity. For a product obtained by freezing a manufactured or processed food, a statement of whether or not the food required heating before consumption [25].

Following specifications standards and testing, methods for foodstuffs, implements, containers and packaging, toys, detergents, and heated meat products must be produced using a method that complies with the following standards:

1) The products must be sterilised using a method that heats them for 30 minutes at a temperature of 63°C as measured at the centre or a method that achieves an equivalent or better effect (for products containing fis that are sterilised after having been packed in air-tight containers, a method that heats them for 20 minutes at a temperature of 80°C as measured at the centre or a method that achieves an equivalent or better effect).
2) After heating and sterilizing, the products must be sufficientl cooled in a hygienic location; when using water; potable running water must be used.
3) Products that have been packed in containers after heating and sterilising must be handled hygienically after being cooled. Fish-paste products (excluding ground fish must test negative for coliform bacilli. Fish sausages and fis ham may not contain silver nitrite in any amount exceeding 0.05 g/kg. The fis used for production must be satisfactorily fresh. The fis used for production must be thoroughly washed prior to processing and must be stored in clean and easy-to-wash impermeable containers made of metal, synthetic resin, etc. Clean preparation apparatus must be used for the filletin and the fillete dressed fis meat must be stored in special-purpose, clean, and easy-to-wash impermeable containers made of metal, synthetic resin, etc. Frozen fis meat used as the raw material for production must be thawed out in a hygienic location. When water is used to thaw it out, sanitary running water must be used. Fish sausage and fis ham must be sterilised using a method that heats them for 45 minutes at a temperature of 80°C as measured at the centre or a method that achieves an equivalent or better effect; specially wrapped kamaboko (white fis meat made into a seasoned paste and steamed) must be sterilised using a method that heats it for 20 minutes at a temperature of 80°C as measured at the centre, or a method that achieves an equivalent or better effect; and other fish-past products must be sterilised using a method that heats them at a steady temperature of 75°C as measured at the centre or a method that achieves an equivalent or better effect [26]. However, this does not apply to ground fish The main points of Japanese legislation are given in Table 27.5.

27.3   Conclusions

In general terms, the EU and US legislations are very similar when definin several categories of production areas. The main difference remains in the method of control to qualify those areas. Even if the production and placing on the market of shellfish fisher , and aquaculture products are subject to Directives 91/492/EEC and 91/493/EEC, those products remain in the so-called "non-harmonized products" category. It means that national rules can be

Table 27.5 Japanese legislation for fish and fishery products

Legislation	Year	Main points	Reference
Bacteriological and Chemical Guidelines for Fish and Seafood	2006	• Fish fillets, shucked shellfish, frozen foods (frozen fish or shellfish) intended to be consumed raw • Oyster intended to be consumed raw • Globefish poison • Shellfish poison • Ciguatera poison • Veterinary drugs residues • Withdrawal periods for shrimp, Japanese flounder, eel • Environmental contaminants should not accumulate in edible parts of the fish beyond unsafe levels of PCB and mercury	[24]
Japan External Trade Organization	2006	• It refers to fish-paste products • It defines the level of temperature and pH of fish-paste products. • It refers to all fish businesses	[25]
Japan External Trade Organization	2006	• It refers to the right methods to sterilise the fish products • Standards for fish-paste product components • Production standards for fish-paste products • Storage standards for fish-paste products	[26]

applied in addition to the EU legislation [27]. The US legislation is based on Procedures for the Safe and Sanitary Processing and Importing of Fish and Fishery Products [18] and Sustainable Fishery Act [19]. The Canadian Fisheries Act refers to fis habitat protection and pollution prevention, and harmful alteration of fis habitat. Fisheries in Queensland are managed under the Fisheries Act 1994. The Act does not establish any right for recreational fisher to fish nor does it impose any specifi obligation on the Minister to have regard to recreational interests when making fisher management decisions. Japanese legislation controls the microbiological counts in fis and fisher products.

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28 Food safety and quality systems (ISO 22000:2005) in the seafood sector

Ioannis S. Arvanitoyannis

28.1 Introduction

In 2004, European Union (EU)-15 countries' exports of fis and fisher products accounted for US$17.1 billion, representing a growth of 51% since 2000. Approximately 80% of these exports were destined for other EU-15 countries [1].

The globalization of the fis trade, coupled with technological developments in food production, handling, processing, and distribution, and the increasing awareness and demand of consumers for safe and high quality food have put food safety and quality assurance high in public awareness and made it a priority for many governments. Consequently, many countries have tightened food safety controls, imposing additional costs and requirements on imports. As early as 1980, there was an international drive towards adopting preventive Hazard Analysis Critical Control Point (HACCP)-based safety and quality systems. More recently, there has been a growing awareness of the importance of an integrated, multidisciplinary approach to food safety and quality throughout the entire food chain. Implementation of this approach requires an enabling policy and regulatory environment at national and international levels with clearly define rules and standards, establishment of appropriate food control systems, and programmes at national and local levels, and provision of appropriate training and capacity building [2].

Seafoods may harbour a number of biological, chemical, and physical hazards, the most prevalent of which are biogenic amines, biotoxins, pathogenic bacteria, and viruses. Some of the largest food poisoning outbreaks have been associated with seafoods. In 1991, more than 300,000 people contracted hepatitis A in Shanghai, in which there were 9 deaths [3]. Around the same time, cholera caused more than 400,000 illnesses and more than 4,000 deaths in Peru; the lightly fermented fish ceviche, was thought to be a major vehicle [4].

Salmonella species are recognized as very important food-borne and water-borne bacteria and the cause of a significan range of illnesses including food poisoning (gastroenteritis), typhoid (enteric fever), paratyphoid, bacteraemia, septicaemia, and a variety of sequelae. To date, almost 2,400 serotypes of *Salmonella* have been identifie and they are of major concern to nearly all sectors of the food industry [5]. The ubiquity of non-typhoid *Salmonella* makes them a persistent contamination hazard to all raw foods, whether they are derived from

animals, fis and shellfish eggs, poultry and game birds, fruits, vegetables, dairy produce, and cereals, etc. [6].

Cold-smoked fis products, although among the highest concern products based on hazard analysis and risk assessment considerations relating to the potential for *Listeria monocytogenes* to be present, have an excellent food safety record, with few outbreaks involving this organism attributed to these traditional products [7]. *Listeria* species do not appear to cause infections in fis and do not have a natural reservoir in fish However, a variety of surveys have shown that they are present in river sediments and water, both freshwater and seawater, and therefore may be present in the raw fis as a contaminant from the aquatic environment [8]. This chapter highlights the application of HACCP and ISO 22000 in fis and seafood. Salmon, crab, and surimi were selected, in order to include three representatives from seafood sector.

28.2 Salmon

While world production of Atlantic salmon has increased, problems related to quality still persist. The temperature in the cold-smoking process never exceeds 28 to 32°C, which limits inactivation of enzymes in salmon tissue [9]. Most of the deteriorative changes that reduce shelf-life of fisher products are due to enzymic activities. The presence of primary food-borne pathogens, such *Listeria monocytogenes* and *Clostridium botulinum* type E, are also of major concern in cold-smoked products [10].

Positive conclusions have been established from various studies conducted on seafoods using high pressure processing that this technology could be applied to seafoods to extend the shelf-life. It achieves this by controlling or inactivating seafood-related spoilage enzymes, modifying texture, stabilizing colour, and inhibiting lipid oxidation [11,12].

The effects of smoking, drying, enzymatic spoilage, feed usage, textural, and biochemical changes during frozen storage of cold-smoked salmon are described by Lakshmanan *et al*. [13]. Important pathogens associated with cold-smoked salmon, such *Listeria monocytogenes* and other spoilage organisms are also described and potential areas for further research are identified The fl w diagram of salmon processing is shown in Fig. 28.1.

The major steps in the preparation of smoked fis are salting (bath or injection of liquid brine or dry salt mixture), cold smoking, cooling, packaging (air/vacuum or modified) and storage. Smoking, one of the oldest preservation methods, combines the effects of salting, drying, heating, and smoking. Typical smoking of fis is either cold (32°C) or hot (70–80°C). Cold smoking does not cook the flesh coagulate the proteins, inactivate food spoilage enzymes, or eliminate the food pathogens, and hence refrigerated storage is necessary until consumption. Gaping is a serious problem associated with cold-smoked salmon. It is characterized by separation of myocommata that makes it difficul to process and sell high value fillet of cold-smoked salmon [14]. The common factors associated with gaping are low pH, smoking process, prolonged storage before freezing, mechanical damage during handling, and lack of proper chilling.

On the fresh salmon production line, the next stage is that of blood removal. This stage is a critical control point (CCP) because the water used must be potable, well-filtere so as to remove harmful substances and micro-organisms, and compatible with the requirements of the Directive 80/778/EC instruction. After blood removal, the fis are classifie with regard to size and quality. Thereafter, fis are placed in heat-insulating packages, which are covered with a transparent membrane suitable for foods. The packing, cooling/conservation,
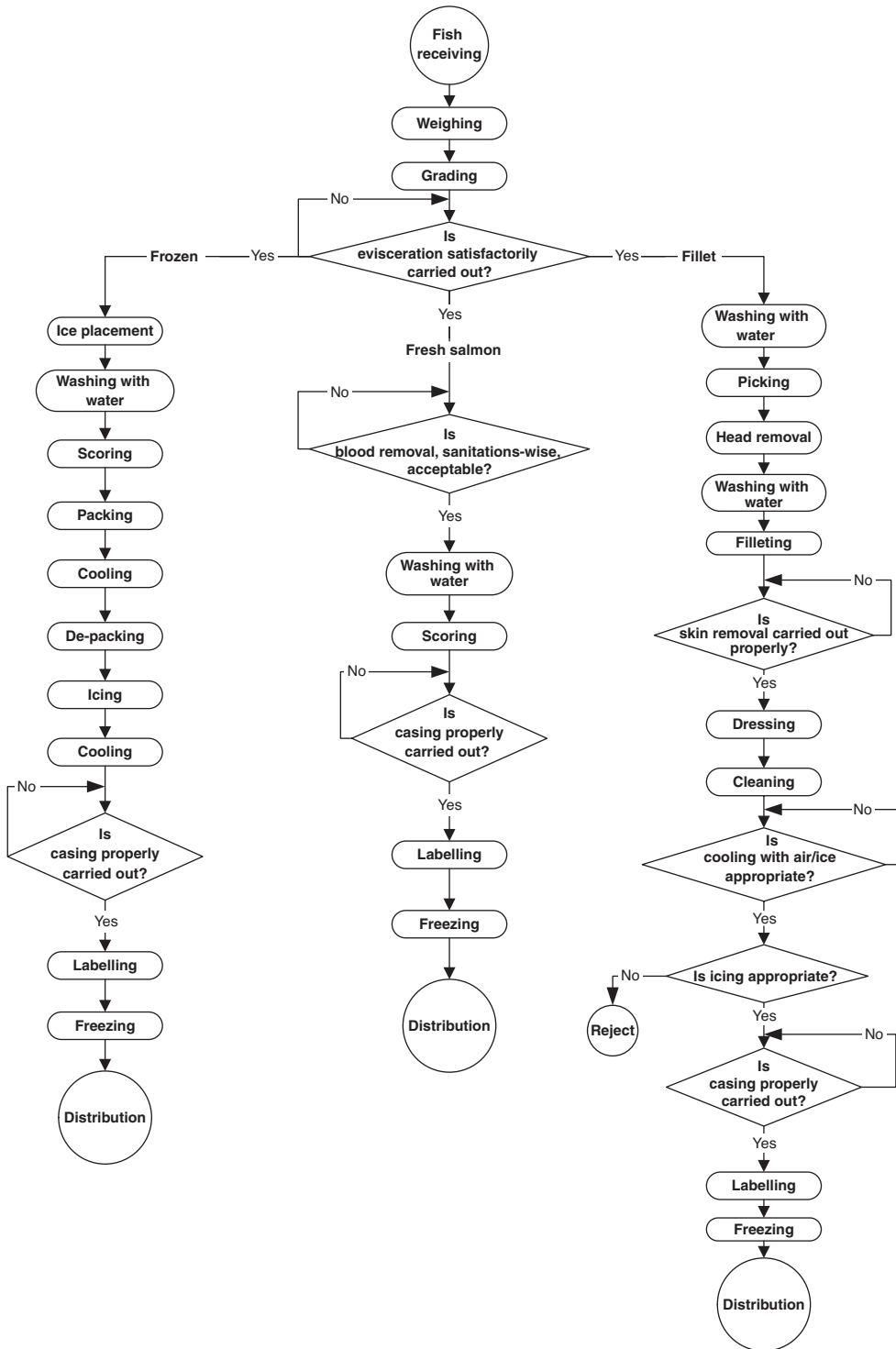
Fig. 28.1 Flow diagram of salmon processing.

which are effected at 4°C, are followed. This stage is a CCP, because the fis will be spoiled if the temperature is higher [15]. For this reason, the checking of the temperature control of cooling installations is necessary at regular periods. The fis are then placed in boxes, which are marked on their external side [16]. At this stage, the personnel must abide by the hygiene rules, so as to avoid contamination of the packaged fish Moreover, labelling must be definit ve and correct. The boxes are placed in the freezer (–18°C) until transported to the market for sale. It is important for the freezing temperature to remain constant during transport, which should be carried out with transportation vehicles operating at –18°C. The questions used to derive a CCP for salmon processing according to HACCP analysis are given in Table 28.1.

Fillet production also begins after evisceration and washing with water to ensure complete blood removal. The same measures as described for the chilled and frozen fis are applied in order to avoid contamination and product denaturation. Sorting and placement of fis on ice in order to be maintained at low temperature is also similar. The next step is the head removal, which is considered to be a CCP, since the risk of entrance of foreign matter (hair) into the fis exists at this point in the process, which is undesirable. Following head removal, washing is carried out to remove any remaining offal. Filleting then follows during which a transverse section is carried out along the vertebral column on both sides of each fish A good filletin process removes almost all the initial microbial load of the fish hence it is easy to produce fillet with no microbes and with a satisfactory shelf-life, even from a fis with a high microbial load. Despite that, this stage is a CCP because fillet might be contaminated by both pathogenic micro-organisms and physical contaminants. The possibility of the appearance of skin, bones, and membranes in fillet also exists. Regular preventative maintenance in conjunction with continuous monitoring of the production line and direct corrective actions in the case of deviations should prevent such phenomena. Product lots with defects get exemptions and may be processed for a second time [17]. The critical control points, hazards, critical limits, corrective actions, and records as well as the ISO 22000 Analysis Worksheet for determination of some prerequisite programmes for salmon processing are summarized in Tables 28.2 and 28.3, respectively. To reduce these hazards, the processing units use good hygiene and control practices. Following removal of the skin from the fillets they may be immersed in a dressing sauce depending on the intended market/use. This is considered a CCP because the sauce could carry a microbial load or pathogenic micro-organisms such as *Escheria coli*. The comparative presentation of CCPs of HACCP and ISO 22000 is given in Table 28.4.

28.3   Surimi

The surimi industry has changed dramatically since 2000. The global decrease in whitefis supply has strengthened the demand for other product forms (fillet and blocks) made from Alaska pollock, while the surimi seafood industry has learned to use lower-quality surimi (lower gel functionality and darker colour) to process surimi products from other species [18]. Mixing surimi with salt, starch, and fl vourings enhances many microbiological hazards. To inactivate non-spore-forming bacteria, adequate thermal processing is required, while it is suggested that the core temperature should be ≥ 65°C for at least 3 minutes [19]. Other common additives might be $NaHSO_3$, cysteine, and ascorbic acid [20].

Fresh fis are preferred for surimi as they contain less blood and gut residues in the tissues and experience less autolysis of the muscle proteins giving a better gel. A uniform

Table 28.1 Questions used to determine CCPs for salmon processing according to HACCP analysis

Processing step	Determination of hazards	Q1 Do preventative control measures exist? (Yes/No)	Q2 Is the step specifically designed to eliminate or reduce the likely occurrence of hazard to an acceptable level? (Yes/No)	Q3 Could there be contamination with identified hazards(s) or could this increase to unacceptable levels? (Yes/No)	Q4 Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to acceptable levels? (Yes/No)	Q5 Is this step a critical control point?
Receipt of fishes	Biological Pathogenic micro-organisms, parasites	YES	NO	YES	NO	CCP1
	Chemical Heavy metals, pesticide residues	YES	NO	YES	NO	
	Physical Extrinsic deformations, bruises	YES	NO	YES	NO	
Weighing	Biological No identified hazard	NO	NO	NO	YES	
	Chemical No identified hazard	NO	NO	NO	YES	
	Physical No identified hazard	NO	NO	NO	YES	
Grading	Biological No identified hazard	NO	NO	NO	YES	
	Chemical No identified hazard	NO	NO	NO	YES	
	Physical No identified hazard	NO	NO	NO	YES	

Process step	Hazard type	Hazard description	Q1	Q2	Q3	Q4	CCP
Evisceration	Biological	Microbial infection, parasites	YES	NO	YES	NO	CCP2
	Chemical	Chemical contamination	YES	NO	YES	YES	
	Physical	Foreign matter	YES	NO	YES	YES	
Blood removal	Biological	Water infected with pathogenic micro-organisms	YES	YES	–	–	CCP3
	Chemical	Infectious agents in water	YES	YES	–	–	
	Physical	No identified hazards	YES	YES	–	–	
Washing	Biological	Microbial contamination	YES	YES	–	–	CCP4
	Chemical	Heavy metals	YES	YES	–	–	
	Physical	Non potable water	YES	YES	–	–	
Sorting	Biological	No identified	YES	YES	–	–	
	Chemical	No identified hazard	YES	YES	–	–	
	Physical	Foreign matter	YES	YES	–	–	

(Continued)

Table 28.1 (Continued)

Processing step	Determination of hazards	Q1 Do preventative control measures exist? (Yes/No)	Q2 Is the step specifically designed to eliminate or reduce the likely occurrence of hazard to an acceptable level? (Yes/No)	Q3 Could there be contamination with identified hazards(s) or could this increase to unacceptable levels? (Yes/No)	Q4 Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to acceptable levels? (Yes/No)	Q5 Is this step a critical control point?
Head removal	Biological Microbial contamination, parasites	YES	NO	NO	–	
	Chemical Chemical contamination	YES	NO	NO	–	
	Physical No identified hazard	YES	NO	NO	YES	
Fillet making	Biological Microbial contamination, parasites	YES	NO	YES	NO	CCP5
	Chemical Chemical contamination	YES	NO	NO	NO	
	Physical No identified hazard	YES	NO	NO	NO	
Skin removal	Biological Microbial contamination, parasites	YES	NO	NO	YES	
	Chemical Chemical contamination	YES	NO	NO	YES	
	Physical Foreign matter	YES	NO	NO	YES	

Process	Hazard					
Dressing	Biological	Growth of pathogenic microorganisms	NO	NO	YES	YES
	Chemical	Industrial chemical compounds	NO	NO	YES	YES
	Physical	None	NO	NO	NO	YES
Hunging	Biological	No identified hazards	NO	NO	NO	YES
	Chemical	No identified hazard	NO	NO	NO	YES
	Physical	No identified hazard	NO	NO	NO	YES
Icing	Biological	Microbial infection	NO	NO	NO	YES
	Chemical	Industrial chemical compounds	NO	NO	NO	YES
	Physical	Foreign matter	NO	NO	NO	YES
Sorting	Biological	No identified hazard	YES	YES	NO	NO
	Chemical	No identified hazard	YES	YES	NO	NO
	Physical	Foreign matter	YES	YES	NO	NO

Table 28.1 (Continued)

Processing step	Determination of hazards		Q1 Do preventative control measures exist? (Yes/No)	Q2 Is the step specifically designed to eliminate or reduce the likely occurrence of hazard to an acceptable level? (Yes/No)	Q3 Could there be contamination with identified hazards(s) or could this increase to unacceptable levels? (Yes/No)	Q4 Will a subsequent step eliminate identified hazard(s) or reduce likely occurrence to acceptable levels? (Yes/No)	Q5 Is this step a critical control point?
Cooling with air or ice	Biological	Growth of micro-organisms, parasites	YES	YES	–	–	CCP6
	Chemical	Rare		–	–	–	
	Physical	Foreign matter	YES	NO	NO	–	
Casing	Biological	Growth of pathogenic micro-organisms	YES	NO	YES	NO	CCP7
	Chemical	Chemical contamination	YES	NO	YES	NO	
	Physical	Foreign matter	YES	NO	YES	NO	
Labelling	Biological	No identified hazard	NO	NO	YES	NO	
	Chemical	No identified hazard	NO	NO	YES	NO	
	Physical	Foreign matter	NO	NO	YES	NO	

Process step	Hazard	Description	Q1	Q2	Q3	Q4	CCP
Freezing	Biological	Microbial growth, parasites	YES	NO	YES	NO	
	Chemical	Rare	YES	NO	YES	NO	
	Physical	Deterioration, quality loss due to slow freezing	YES	NO	YES	NO	
Distribution	Biological	Microbial growth and contamination	YES	NO	YES	NO	CCP8
	Chemical	Chemical contamination	YES	NO	YES	NO	
	Physical	Product destruction	YES	NO	YES	NO	

Table 28.2 Critical control points, hazards, critical limits, corrective actions, and records for salmon processing

Critical control point (CCP)	Significant hazards	Critical limits measure	Control				Corrective action	Records	Verification
			What	How	Frequency	Who			
Whole salmon receiving	Pathogenic micro-organisms from breeding unit Biotoxins Histamine formation Environmental infectious agents and pesticides Heavy metals	Determined by national regulations <100 ppm histamine 2 mg per 100 gm body weight	Determination of the cultivation area	Chemical and microbiological analysis Macroscopic control Questionnaire for the location of the breeding unit	For every supplier	Production Supervisor Quality Control Staff	Stop fishing authorized by control agencies Remove fishes Stop working with these suppliers	Receiving records	Review, control and record correction 1 wk following incident occurrence
Evisceration	Biological danger: Pathogenic micro-organisms	No presence Process should not last >1 h Possible suspicion for contamination should place the product on hold	Determination of the possible consequences in fishes from wrong application of the technique	Macroscopic control Temperature recording meters Sampling for microbiological control	For every lot produced in 1 h	Production Staff, Production Supervisor	Good hygiene practice, tools disinfection following evisceration	Processing	Review of the evisceration technique and macroscopic control of fishes to detect any contamination

	Biological hazard								
Blood removal	Biological hazard: Pathogenic micro-organisms	No presence Process should not last >1 h Possible suspicion for contamination should place the product on hold	Determination of the possible consequences in fishes from wrong application of the technique	Macroscopic control Temperature recording meters Sampling for microbiological control	For every lot produced in 1 h	Production Staff Production Supervisor	Good hygiene practice, good filtration of washing water	Processing	Review of the technique and macroscopic control of fishes to detect any contamination
Filet making	Pathogenic micro-organisms	No presence Process should not last >1 h Possible suspicion for contamination should place the product on hold	Determination of the possible consequences in fishes from wrong application of the technique	Macroscopic control Temperature recording meters Sampling for microbiological control	For every lot produced in 1 h	Production Staff Production Supervisor	Good hygiene Temperature control	Processing records	Controlled hygiene and sanitation Temperature measurements
Chiling/Freezing	Parasites	Frrezing at −18°C Cooling at 4°C for 24 h	Temperature of cooling air Time period of ice	Macroscopic fish control Temperature recording meter	Continuous, freezing cycle	Fridges and Freezers Operator	Maintenance of fridges and freezers Repeat of the process	Temperature control chart for each freezing cycle	Review, control and correction of the records in 1 wk from preparation Recording of the daily temperature inside the fridges

Table 28.2 (Continued)

Critical control point (CCP)	Significant hazards	Critical limits measure	Control					Records	Verification
			What	How	Frequency	Who	Corrective action		
Casing/marking	Growth of pathogenic micro-organisms Chemical contamination Inadequate marking, weight, dehydration	Products should not be exposed to temperatures over 4°C for >3 h	TTI label per packaged unit	Macroscopic control during packaging and marking before freezing	Every packaged unit	Packaging operator	Placement of labels 1 h after casing ready to be stored	Verification TTI records	Internal activation trials for new TTIs, and recording of validation sheets for each order given by the suppliers
Distribution	Possible presence of Clostridium botulinum toxin in the packaging with reduced oxygen, if the product is not transported in the right packaging	Time and temperature should not exceed the limit for thermal de-composition of the product	Colour changes showing thermal de-composition	Macroscopic control during storage and distribution	Before storage, distribution and acceptance of packaged product	Packaging operator	Reject/destruct any packaged product exceeding the critical TTI limit	Verification TTI records	Packaging records before distribution showing any product being rejected due to TTI changes

Table 28.3 ISO 22000 Analysis Worksheet for determination of some prerequisite programs for salmon processing

Processing step	Are the technical infrastructure and the preventative maintenance program adequate? (Yes/No)	Is it feasible to evaluate them? (Yes/No)	Do they contribute in the control of recognizable food safety hazards? (Yes/No)	Does the effectiveness of the remaining control measures depend on them? (Yes/No)	Is it a prerequisite program? (Yes/No)
Receiving of fishes	YES	YES	NO	NO	NO
Weighing	YES	YES	NO	YES	YES
Grading	YES	YES	NO	YES	YES
Evisceration	YES	YES	NO	NO	NO
Blood removal	YES	YES	NO	NO	NO
Washing	YES	YES	NO	YES	YES
Sorting	YES	YES	NO	YES	YES
Head removal	YES	YES	NO	YES	YES
Filet making	YES	YES	NO	NO	NO
Skin removal	YES	YES	NO	YES	YES
Dressing	YES	YES	NO	YES	YES
Hanging	YES	YES	NO	YES	YES
Icing	YES	YES	NO	YES	YES
Sorting	YES	YES	NO	YES	YES
Cooling with air or ice	YES	YES	NO	NO	NO
Casing	YES	YES	NO	NO	NO
Labelling	YES	YES	NO	YES	YES
Freezing	YES	YES	NO	YES	YES
Distribution	YES	YES	NO	NO	NO

Table 28.4   Comparative presentation of CCPs of HACCP and ISO 22000 in conjunction with PRP for salmon processing

Process step	HACCP CCPs	Prerequisite program according to ISO 22000	ISO 22000 CCPs
Receiving of fishes	1	NO	1
Weighing	–	YES	–
Grading	–	YES	–
Evisceration	2	NO	2
Blood removal	3	NO	–
Washing	4	NO	–
Sorting	–	YES	–
Head removal	–	YES	–
Fillet making	5	NO	3
Skin removal	–	YES	–
Dressing	–	YES	–
Hunging	–	YES	–
Icing	–	YES	–
Sorting	–	YES	–
Cooling with air or ice	6	NO	4
Casing	7	NO	–
Labelling	–	YES	–
Freezing	–	YES	–
Distribution	8	NO	–

size of fis is important for consistent yields from deboning/mincing machines, and fis with a good fles to frame ratio may give overall better yields of mince. Water quality is important for preventing microbial growth. Salt is added to lower the water activity (a_w), but its concentration should be more than 0.2%, otherwise actin and myosin may be solubilized. The surimi must be stored below 20°C [21].

The addition of alkali in the surimi wash water produces a higher-quality product than just using water. Various concentrations of sodium bicarbonate can be added in one or more of the wash steps to increase the pH. Sodium chloride is sometimes added. It has been suggested that gelation is improved after this type of washing process because the "solubility of the sarcoplasmic proteins" is increased and there is a "decreased rate of denaturation as the muscle pH is increased" [22].

Surimi seafoods are often vacuumed-packed and sold under refrigerated storage. The potential hazards for surimi seafood can include the inclusion of metal fragments and the existence of human pathogens, such as *Listeria monocytogenes* and *Clostridium botulinum*. Therefore, CCPs for eliminating or reducing these hazards from surimi seafood include the pasteurization process, rapid cooling, low-temperature storage, and metal detection [23].

Pasteurization is a heat process designed to eliminate targeted bacterial pathogens and reduce total populations of spoilage bacteria in products. Although bacterial spores usually survive the heat process, a properly pasteurized product should contain a minimal amount of spoilage bacteria and be free of pathogens. Rapid cooling of pasteurized products will prevent the germination of bacterial spores and the growth of spore-forming bacteria such as *Bacillus* and *Clostridium* species. Pasteurized surimi seafood should be cooled from 60°C to less than 21.1°C within 2 hours and to less than 4.4°C within another 4 hours to prevent

spore germination as well as retard the growth of spoilage bacteria [24]. Vacuum packed surimi seafoods that are sold under refrigerated storage should be kept at temperatures below 3°C to prevent the growth and toxin production of non-proteolytic types of *Clostridium botulinum*. Foreign objects such as metal fragments can cause injury to consumers and should be considered possible hazards associated with surimi seafood production. Metal fragments can be produced through metal-to-metal contact, especially during mechanical cutting or blending operations during surimi and surimi seafood production [6].

28.4 Crabs

Fisheries for two commercial crab species in Australia (Spanner and Blue Swimmer crabs) are found in Queensland, New South Wales, and Western Australia. These are caught in both estuarine and marine waters, using baited tangle nets, or in traps, hoop nets, or drag nets.

When moving as large aggregations, Spanner crabs are also occasionally caught as a by-product of dermesal otter trawling operations. Cadmium has been identifie as a food safety hazard associated particularly with spanner crabs (*Ranina ranina*).

Crabs inhabit similar estuarine and marine environments to prawns, and are potentially exposed to the same environmental hazards, both chemical and microbial. Raw and frozen product is also subject to similar processing and similar potential hazards. Endogenous bacteria that are human pathogens (e.g. *Vibrio*s and *Aeromonas hydrophila*) and environmental contaminants (e.g. arsenic and mercury) are potential hazards. Post-harvest handling, processing, transport, and storage potentially introduce and allow outgrowth of human enteric pathogens (*Escherichia coli*, *Campylobacter*, *Shigella*, *Yersinia*, and *Salmonella* spp., and noroviruses and hepatitis A virus) and *Listeria monocytogenes*. However, as crab is generally sold either as live or raw frozen product, and is generally cooked thoroughly just before eating, concerns regarding microbiological contamination of cooked product prior to consumption are less relevant than for cooked prawns [25].

The concentrations of cadmium, copper, mercury, and zinc were determined in muscle (body, claw, and leg), hepatopancreas, and gill tissues of *Pseudocarcinus gigas*, an exceptionally large, long-lived, and deep-dwelling crab species. The accumulation patterns observed are discussed in terms of both intra- and interspecies variations, with particular attention to the possible consequences of the extreme size and depth range of *Pseudocarcinus gigas*. Metal concentrations did not depend significantl on sex of the crab. The concentrations of mercury and zinc in muscle tissue increased with crab size and were high compared to other crab species. The concentrations of cadmium and copper present in edible tissues were not especially high compared to other crab species, but the concentration of cadmium in the hepatopancreas is of dietary concern [26].

28.5 Conclusions

The HACCP system, which is science based and systematic, identifie specifi hazards and measures for their control to ensure the safety of fis and seafood. A CCP is a step at which control can be applied to prevent or eliminate a fis and seafood safety hazard or reduce it to an acceptable level. Prerequisite programmes are the foundation of the HACCP plans and must be adequate and effective. More and more fis and fisher products are traded internationally.

International standards such as ISO 22000 are crucial for giving fina consumers confidenc that the products they buy are safe, regardless of where they have been produced.

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Part III
Health applications of seafood

29 Health benefits associated with seafood consumption

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29.1 Introduction

The use of fis and shellfis in human nutrition is well documented since ancient times in archaeological settlements as well as in ancient civilizations. However, fis processing and international trade gained enormous importance only in the 20th century [1].

The consumption of fis and fish-der ved products has increased over recent decades in many countries, especially between 1980 and 2001, as a result of higher living standards and the good image of seafood among consumers. The world average use of fis products reached 16.6 kg per capita in 2005 [2], but it is unevenly distributed around the globe, with marked continental, regional and national differences as well as income-related variations. Per capita apparent annual fis consumption can vary from less than 1 kg to more than 100 kg.

Seafood encompasses a wide range of wild and farmed animals and seaweeds, in which fish crustaceans, and molluscs are the most important groups, both due to the high diversity of species and its use as food. The traditional view of seafood as a source of high-quality animal protein to fulfi the basic food requirements has shifted, and a significan part of the actual demand is related to its peculiar structure and physical, chemical, and sensory attributes. In fact, these characteristics associated with a high number of available species, has led seafood to play a particular role in a balanced diet as well as in modern gastronomy. On the other hand, the relevance of seafood in the diet to diminish the increased incidences of cardiovascular, cancer, and inflammator diseases and to improve consumer's well-being has been successfully supported by the results of a high number of epidemiological studies and meta-analyses. This chapter presents relevant information on nutritional value and some benefit associated with the consumption of seafood.

29.2 Nutritional value

The chemical composition of fis products varies greatly among species and from one individual fis to another, depending on age, sex, environment, and season. Proteins and lipids are the major components, whereas carbohydrates are usually detected at very low levels (<0.5%) [3]. Vitamin content is comparable to that of mammals, except for vitamins

A and D, which are found in large amounts in the meat of fatty species and in the liver of lean fish such as cod and halibut. Fish meat is a particularly valuable source of minerals, namely calcium and phosphorus as well as iron, copper, and selenium. In addition, saltwater fis is an excellent source of iodine.

29.2.1 Protein

Proteins are important for the growth and development of the human body, maintenance and repairing of damaged tissues, and for production of enzymes and hormones required for many body processes. For most seafood species, protein content ranges between 10 and 25%, with an average of 17 to 100 g, which accounts for 80 to 90% of the energy provided per 100 g of lean species [4]. The protein found in seafood is of good quality due to its high digestibility, and the specifi amounts and relative proportions and availability of essential amino acids. The amount of connective tissue in fis and shellfis muscle is quite low and it softens and dissolves more readily when heated compared to the connective tissue of land animals, making seafood meat easy to chew. Almost all species are well balanced with respect to their essential amino acids. The predominant amino acids are usually lysine and leucine and, within the nonessential, aspartic and glutamic acids are the most abundant. Very often the amount of essential amino acids is greater than that in the standard protein (32–100 g protein) and values regularly referred to in the literature for the chemical score, biological value, and protein efficien y ratio. Protein digestibility and corrected amino acid score are also good indicators of the quality of fis proteins [5,6].

29.2.2 Lipids

Lipids perform several important biological functions for living organisms, namely storage and transport of energy, formation of cell membranes, maintenance of their structural integrity, and prostaglandins synthesis and transport of fat-soluble vitamins. Fish lipid content varies, depending on the species as well as on the season but, in general, fis have less fat than red meats. Fat content ranges widely from 0.2% to almost 30%. Contrary to terrestrial animals, in which most lipids are generally deposited in adipose tissue, fis have lipids in the liver, muscle, and perivisceral and subcutaneous tissues. According to the fat content, fis products are generally classifie into three categories. For instance, Atlantic salmon, European sardine, herring, mackerel, and eel have more than 10% muscle fat and are considered fatty, whereas lean species, such as hake and cod, have less than 1% of muscle fat. Other species, such as trout, sea bass, or sea bream, are classifie as intermediate because their muscle lipid content accounts for 5 to 10% of their wet weight. Regarding farmed fish lipid content can vary widely depending on the farming conditions and composition of the feed. Fish lipids are composed of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA), whose proportions and amounts vary considerably from one species to another (Table 29.1) [4]. As a rule, the fattest species contain more long-chain omega-3 (n-3 or ω-3) PUFA than the leaner species; the amount of SFA, in percentage, is almost constant in most species. In the majority of species, PUFA are the dominant group; however, there are some exceptions, for instance meagre and silver- and black-scabbard fish where the content of MUFA is higher than that of PUFA. In general, palmitic acid (16:0) is the most relevant within the SFA group, oleic acid (18:1 n-9) is the dominant in MUFA, and eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) present the highest amounts in PUFA [7].

Table 29.1 Typical nutritional data of molluscs, crustaceans, and fish products. Adapted from Nunes et al. [4], with permission of Wegeningen Academic Publishers

Nutritional data (per 100 g of edible part)	Grooved carpet shell Raw	Common octopus Raw	Common octopus Boiled	Norway lobster Raw	Atlantic salmon Raw	Atlantic salmon Boiled	Atlantic salmon Grilled	European hake Raw	European hake Boiled	European hake Fried	Sardine Raw	Sardine Grilled	Sardine Canned
Energetic value (kcal)	58.1	77.4	116.5	93.8	266.7	278.7	315.4	73.9	118.9	163.7	187.1	197.7	210.7
Protein (g)	11.7	15.6	23.7	20.9	16.2	20.7	23.8	17.0	20.1	21.7	17.9	24.1	24.0
Total fat (g)	0.9	1.2	1.3	0.5	21.9	21.1	23.7	0.7	3.7	7.1	10.9	9.2	12.7
16:0 (mg)	138.4	177.4	189.7	60.1	2687.7	2450.1	2753.5	89.7	549.4	507.7	1695.3	1487.6	1994.7
Total SFA (mg)	223.5	265.9	282.9	89.0	4291.3	4049.4	4487.6	142.8	856.6	778.1	2745.9	2396.3	3001.4
18:1 (mg)	25.2	40.8	43.6	66.9	3809.7	2450.1	2821.6	55.1	331.9	1658.6	979.8	742.1	4375.7
Total MUFA (mg)	119.7	90.1	96.6	87.1	10037.3	7824.5	8747.4	110.3	650.0	1790.4	2557.5	2069.4	5581.9
18:2 n-6 (mg)	5.0	4.8	0.9	3.9	691.2	603.9	695.0	7.3	40.6	3445.4	104.5	85.8	423.8
20:5 n-3 (mg)	58.6	196.5	211.0	57.1	1172.1	1629.9	1800.0	66.0	371.4	91.9	1671.8	1287.9	791.7
22:6 n-3 (mg)	54.9	225.3	239.2	76.8	1772.6	2326.3	2593.8	155.3	980.1	258.6	1169.4	1334.2	1255.7
Total PUFA (mg)	255.7	560.0	591.3	155.1	5148.2	6590.6	7359.0	273.4	1644.8	3860.8	4071.0	3493.8	2806.0
Total PUFA n-3 (mg)	190.0	496.9	525.6	139.4	4326.4	5622.6	6255.4	246.9	1491.7	388.4	3753.3	3245.9	2307.7
Total PUFA n-6 (mg)	65.7	63.1	65.7	15.7	765.6	968.0	1103.6	26.6	153.2	3472.4	317.7	247.9	498.3
Cholesterol (mg)	44	64	105	68	40	na	na	19	28	25	28	38	na
Calcium (mg)	51	13	26	72.0	12	61	68	15	29	54	70	67	445
Phosphorus (mg)	178	165	185	216	209	216	322	219	230	303	296	307	637
Magnesium (mg)	103	43	49	40.5	23	26	40	26	32	43	29	35	42
Iron (mg)	8.5	0.7	0.5	0.4	0.5	0.3	0.4	0.5	0.5	0.7	1.7	1.9	3.0
Sodium (mg)	244	259	178	444	38	148	783	69	169	1344	65	390	187
Potassium (mg)	78	236	164	413	301	234	408	408	373	595	404	496	369
Manganese (mg)	0.65	<0.02	0.04	0.10	<0.02	0.02	0.04	<0.02	<0.02	0.03	<0.02	<0.02	0.21
Copper (mg)	0.18	0.21	0.50	2.50	0.06	0.06	0.04	<0.03	0.03	<0.03	<0.03	0.11	0.15
Zinc (mg)	2.1	1.3	2.4	4.5	0.5	0.8	0.9	0.7	0.8	0.8	1.7	1.2	2.5
Chloride (mg)	347	438	258	na	46	225	1125	85	195	1592	152	740	327
Vitamin A (µg)	na	2.7	6.7	8.3	33	65	70	2.8	5.3	4.3	12	9.0	9.0
Vitamin E (mg)	na	0.73	2.1	2.2	4.0	5.3	4.3	0.24	0.45	na	0.025	0.7	1.5
Vitamin D (µg)	na	0	0	na	11	11	9.2	5.6	5.2	7.0	17	11	8.8
Vitamin B_1 (mg)	na	0.022	<0.018	na	0.18	0.17	0.19	0.019	0.018	0.036	0.018	0.049	<0.02
Vitamin B_2 (mg)	na	0.042	0.044	na	0.041	0.081	0.12	0.044	0.035	0.065	0.14	0.19	0.04
Vitamin B_6 (mg)	na	0.067	0.046	0.1	0.45	0.34	0.21	na	na	na	0.41	0.30	0.1
Vitamin B_{12} (µg)	na	1.3	1.7	1.4	na	na	na	0.63	0.36	0.83	10	9.3	na
Folate (µg)	na	12	13	13.5	10	8.4	10	27	23	28	24	31	21
Niacine (mg)	na	1.3	2.5	2.2	na	3.0	4.4	1.2	1.0	1.8	6.2	8.4	6.0

Cholesterol is an important lipid component in cell membranes, and the body uses it in building a number of hormones and vitamin D. This compound has been the subject of several studies for its role in clogging arteries and thus contributing to heart disease and stroke. Cholesterol in many marine species is the main sterol, accounting for more than 90% of all sterols, while in some shellfis species it might be present at percentages that can be as low as 25% [8]. Cholesterol levels are not significan in most seafood products and those found in fis and a large number of shellfis species are between 24 and 85 mg/100 g (Table 29.1). In bivalve molluscs, phytosterols are also present, coming from microalgae and sediments [9–11]. However, cephalopods usually contain higher levels, for example, European squid has approximately values near 140 mg cholesterol/100 g tissue [4]. Nevertheless, according to some authors [12], the presence of high amounts of taurine in these species helps to reduce cholesterol absorption. This hypocholesterolemic effect of taurine is due to the enhancement of cholesterol degradation and the excretion of bile acid, as referred to by Yokogoshi *et al.* [13]. As a rule, cholesterol contents in wild and farmed fis species are not significantl different.

29.2.3   Minerals and vitamins

Minerals help the body's cellular activity, particularly in enzyme action, muscle contraction, nerve reaction, and blood clotting. For most fis species the order of prevalence is potassium → chloride or phosphorus → sodium → magnesium → calcium → iron → zinc → copper → manganese (Table 29.1). Canned fis products, such as sardines, smelts, and salmon are especially valuable sources of calcium due to the presence of soft bones. Oysters and crustaceans are usually good sources of zinc; oysters, bluefish and shrimp are rich in iron; oysters, crabs, and lobster contain relevant levels of copper. In general, all seafoods are important sources of selenium and iodine, particularly relevant in wild species. Fresh seafood is low in sodium, but in some processed products such as when smoked, cured, and surimi, the content of this mineral could be slightly higher.

Among their many functions, vitamins enable the assimilation of carbohydrates, proteins, and fats. They are also critical in the formation of blood cells, hormones, and neurotransmitters. Fish products usually are not a predominant source of vitamins; however, levels of vitamin B, particularly niacin, B_{12} and B_6, are comparable to those of other foods with high protein content, and some fatty species supply reasonable amounts of vitamins A and D. These vitamins are found especially in fis liver oils. However vitamins are also present in flesh such as α-tocopherol, which could attain 4 mg/100 g in salmon.

29.3   Effect of cooking on nutritional value

The type of cooking method may affect some nutritional components (Table 29.1). Moisture content usually decreases during the cooking process and the size/shape, thickness, and the fis species also influenc such a decrease. Consequently, the proportion of solids increases and the amounts of certain nutrients could be higher in cooked products. Usually, frying leads to a higher water loss associated to the absorption of oil, resulting in an increased fat content. Nevertheless, oil absorption seems to be higher when the fat content of the product is lower. As expected, the fatty acid profil in fried products is influence by the composition of the vegetable oil used. Relative to minerals and vitamins, there is not a common trend. Usually, sodium content increases due to the salt added before cooking and some vitamins

are destroyed while the content of others is not significantl changed. In general, boiling and grilling are quite satisfactory in terms of nutrient keeping.

29.4   Health benefits of seafood

The health benefit of fis products have been claimed for many years and they seem to be strongly correlated to the quality of proteins and the presence of high amounts of n-3 PUFA. Such benefit have been described in several papers, reviews, and reports, those associated with the role of these fatty acids in the prevention of several diseases being the most thoroughly documented.

29.4.1   Essential n-3 fatty acids

The long chain n-3 PUFA, such as EPA and DHA, are very important from a nutritional point of view and can be mainly found in marine fis products. However, it is important to take into account that both fatty acids are not produced by fish but by unicellular marine microalgae that are consumed by other marine species [14] and accumulated through the trophic chain.

Alpha-linolenic acid (ALA, 18:3 n-3) is the essential fatty acid precursor of the n-3 series synthesized in plant organisms using Δ 12- and Δ 15-desaturases [14,15]. However, ALA cannot be synthesized by animals due to the lack of these desaturase enzymes [16] and its essential importance for mammalian was recognized [17]. The conversion of ALA into EPA and EPA into DHA occurs in healthy human adults at a limited rate that can attain 5% in the case of EPA production and only 0.05% for DHA [18,19]. Such rates confir   the importance of the inclusion of these n-3 PUFA in the diet.

29.4.2   Cardioprotector effect/coronary heart disease (CHD)

The association of long chain n-3 PUFA and cardiovascular disease (CVD) was established from the prior observations of low CHD mortality in Eskimos from Greenland, despite their high fat intake [20,21]. Pioneer research studies from the 1970s on the Greenland Inuit indicated that the intake of n-3 PUFA (fish seal, and whale meat) reduced the risk of myocardial infarct, and researchers proposed that the mechanism associated with this effect was the reduction of thrombosis risk [22].

These finding led to a high number of research works trying to establish a relationship between n-3 PUFA intake and CVD. A few of these studies were not conclusive but the vast majority pointed out to a positive role of n-3 PUFA in the prevention of CVD [23].

A part of the effects of dietary n-3 PUFA on CVD is explained by the traditional lipoprotein risk factors associated to the blood levels of total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triacylglycerols (TAGs), as well as by other mechanisms relating to haemostasis, lipid peroxidation and oxidative stress, and inflammator processes, with endothelial function also being involved [24].

Many systematic reviews on observational studies, randomized controlled trials, and clinical, animal, and *in vitro* studies suggest that the regular intake of n-3 PUFA protects against coronary artery and sudden death [25]. Moreover, a meta-analysis based on primary and secondary CHD prevention showed a significan decrease of all-cause mortality risk [18,26]. Another study carried out over two years confirme that men with a previous myocardial infarction receiving daily fis oil capsules (900 mg EPA+DHA) or 200 to 400 g of fatty fis

per week containing 500 to 800 mg n-3 PUFA per day, presented a reduction of 29% in global mortality and 33% in cardiac mortality [27]. GISSI study [28] based on a high number of myocardial infarcts survivors, supplemented with a daily dose of 850 mg of EPA and DHA showed a reduction of 21% in global death and 45% in sudden death. Moreover, dietary n-3 PUFA seems to stabilize the myocardium electrically, resulting in reduced susceptibility to ventricular arrhythmias, thereby reducing the risk of sudden death. The intake of n-3 PUFA was also positively related with the prevention of cardiac arrhythmias in animal model studies, due to the development of a non-fatal ventricular fibrillatio as well as ventricular tachycardia and ventricular premature beats [23].

From a meta-analysis across 11 cohort studies [29], it was concluded that fis consumption is inversely associated with fatal CHD, and mortality from CHD may be reduced by eating fis once per week or more. Further studies [30–32] also confirm the positive effects of fis n-3 PUFA consumption.

Controversial results relating n-3 PUFA intake and stroke incidence were presented in a review by Sidhu [23]. Later, Psota *et al*. [32] pointed out a beneficia association between the ingestion of these fatty acids and stroke reduction in humans.

The protein component of fis also influence the concentration of lipid plasma constituents. Thus, several papers were published with studies on animals. The studies in rabbits [33,34] conclude that dietary proteins act synergistically with dietary lipids to regulate cholesterol metabolism and cod proteins induced a decrease of the very low-density lipoprotein (VLDL) cholesterol level in plasma. It was also concluded that cod proteins increased HDL cholesterol and reduced TAG concentration in plasma, which was accompanied by an increase in lipoprotein lipase (LPL) activity and reduction in VLDL cholesterol levels. The studies in rats concluded that cod protein decreased plasma TAG and cholesterol concentration [35]. The increase of LPL activity in the adipose tissue of rats fed with cod proteins was observed [36]. Demonty *et al*. [37] showed that both cod protein and menhaden oil exert independent and beneficia effects on lipid metabolism in rats. They also demonstrated that the combination of cod protein and fis oil resulted in 50% lower plasma TAG compared with the casein-beef tallow mixture.

The results obtained in human studies showed that the consumption of lean white fis by postmenopausal women induced higher concentrations of total and HDL cholesterol, LDL apolipoprotein B (Apo B), and sex hormone-binding globulin than other animal protein sources [38]. In another work with humans, the consumption of fis protein from lean white fis induced lower plasma VLDL TAG and higher concentrations of LDL TAG and LDL Apo B in premenopausal women [39]. The results of the study by Lacaille *et al*. [40] suggested that fis proteins may be partly associated with the variations in plasma sex hormones status and plasma lipoprotein lipase activity in normolipidemic men. The effects of the incorporation of lean beef, poultry, and lean fis into a diet with a high PUFA:SFA ratio and high fibr content on lipoprotein profile in hypercholestorolemic men were studied by Beauchesne-Rondeau *et al*. [41]. The lean fis diet had the added benefi of improving HDL_2 cholesterol level and significantl increased the ratio of HDL_2 to HDL_3 cholesterol more than the lean beef and poultry diets.

29.4.3   Hypertension

A meta-analysis across 31 studies with fis oil supplementation, based on a dose response effect of n-3 PUFA on blood pressure, was referred to by Morris *et al*. [42], showing a decrease of 0.66 mm Hg in systolic and 0.35 mm Hg in diastolic pressure per gram of n-3

PUFA. Nevertheless, the hypotensive effect of these fatty acids was more evident in subjects with clinical atherosclerosis or hypercholesterolemia. Subsequent works pointed out a more active role in blood pressure reduction of DHA compared with EPA [43], inhibiting the renin–angiotensin system.

29.4.4   Diabetes

The lower incidence of non-insulin-dependent diabetes mellitus (NIDDM) in populations consuming large amounts of fis was reported by Kromann and Green [44]. The consumption of n-3 PUFA has been associated with a low incidence of diabetes, improving the insulin sensitivity [23]. A recent work in n-3 PUFA consumption during an energy restriction study pointed out the importance of n-3 PUFA consumption for the improvement of insulin sensitivity and possibly for the prevention of type-2 diabetes, with a positive effect on insulin resistance in young overweight individuals, independent from changes in body weight, TAG, erythrocyte membrane, or adiponectin [45].

Some epidemiological studies [46] on a population of lean fis eaters suggested that a fis constituent other than n-3 PUFA protected against the development of impaired glucose tolerance and NIDDM. In this respect, some studies with rats [47–49] evaluated the role of dietary cod proteins in the regulation of insulin sensitivity. They demonstrated that cod proteins improved glucose tolerance and appeared to involve, at least in part, a direct action of amino acids on insulin-stimulated glucose transport in skeletal muscle cells. It was also concluded that these proteins normalized the activation status of the phosphatidylinositol (PI) 3-kinase/Akt pathway, which was associated with increased translocation of glucose transporter type 4 (GLUT4) to the T-tubules. The metabolic effect of dietary proteins on insulin and glucose responses in healthy women was investigated by von Post-Skagegård *et al*. [50], who concluded that a cod protein meal, compared with milk or soy protein meal, lowered insulin levels and reduced the insulin to C-peptide and insulin to glucose ratios. Ouellet *et al*. [51] demonstrated that cod protein improved insulin sensitivity compared with other animal proteins in insulin-resistant men and women. According to these authors, this beneficia effect could be attributed to the specifi amino acid composition of these proteins, with lower branched-chain amino acids (valine, leucine, and isoleucine) and the higher arginine content of the cod protein diet compared with other animal protein diets. It is also mentioned that taurine, whose content is about three to four times greater in white fis than in beef or pork, also improved insulin sensitivity. The influenc of dietary intake of proteins from different sources on the occurrence of microalbuminuria in type-1 diabetic patients was studied by Möllsten *et al*. [52]. The major finding of this control study indicated that a diet including a high amount of fis protein (∼9.3 g of fis protein per day) lowered the risk of microalbuminuria in young type-1 diabetic patients.

29.4.5   Cancer

In a systematic review about the effect of n-3 PUFA on cancer risks by McLean *et al*. [53], a delay in the onset of some cancer types (breast, colorectal, lung, and prostate) and the intake of these fatty acids was found. In the case of aero-digestive, bladder, lymphoma, ovarian, pancreatic, and stomach cancer, no association between n-3 PUFA intake and cancer incidence was established. Chapkin *et al*. [54] showed that n-3 PUFA suppressed the mediating inflammator Th 1 cells that are linked to the occurrence of colon cancer. In a study by Istfan

et al. [55], it was hypothesized that n-3 PUFA and vitamin D had the potential to delay the progression of prostate cancer cells.

29.4.6   Other effects

The consumption of n-3 PUFA seems to reduce the risk of depression, postpartum depression, bipolar disorder, schizophrenia, and humour fluctuation [56]. The positive effect of EPA in schizophrenia treatment was demonstrated when it was added to usual antipsychotic agent [57]. Other studies showed a positive role of n-3 PUFA in the control of rheumatoid arthritis, prevention of osteoporosis [58], development of the nervous system, improvement of photoreception, and reproductive system [23], as well as in weight loss [59].

29.5   Conclusions

Seafood is an important source of nutrients, which are fundamental for a balanced diet. Moreover, the recognized beneficia health effects of fis lipids and proteins make fis a food item especially recommended for human health and well-being. Daily recommendations for n-3 PUFA intake were established based on data related with the prevention and the treatment of CVD. The consumption of two fis meals a week or at least a mean level of 500 mg of EPA+DHA per day are actually strongly recommended by several health authorities [60,61]. In the case of secondary prevention, a double dose of 1 g per day is advised [62].

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30 A new approach to the functional improvement of fish meat proteins

Hiroki Saeki

30.1 Introduction

Fish and shellfis are important protein sources and are widely used as raw material for preparation of processed seafood. Fish meat is highly nutritious, and its myofibrilla (Mf) protein has excellent functional properties, such as emulsion- and gel-forming abilities, and water-holding capabilities. Various technologies for dealing with fis meat, such as freeze-drying, spray-drying, extrusion cooking, and high-hydrostatic-pressure processing, have been developed and applied to the seafood industry. However, fis Mf protein is thermally and chemically less stable than that of other vertebrates, and the food functionality is impaired easily as protein denaturation progresses. Therefore, the suppression of protein denaturation during the storage of materials and food processing is important to the manufacturing of high-quality processed seafood. Processing technologies, such as low-temperature storage to keep fis fles close to freezing (partial freezing), mixing with edible cryoprotectants, and rapid neutralisation of meat by washing with alkaline solution, have been developed for decreasing protein denaturation during processing.

Protein glycosylation is an effective method for improving the functional properties of proteins, and various techniques are available to prepare synthetic glycoproteins [3–5,8,18]. Recently, the focus has been placed on the neoglycoprotein-synthetic system using the Maillard reaction [14] as an effective method to improve the functional properties of food proteins. The protein glycosylation system is superior to other types of chemical modificatio for food proteins because it proceeds under mild and safe conditions without the use of chemicals. Food proteins, such as ovalbumin [14], β-lactoglobulin [9], phosvitin [26], lysozyme [36], and protamine [21], have been conjugated with various reducing sugars through the Maillard reaction to improve their functional properties such as thermal stability, emulsion-forming ability, antioxidant system to fis meat protein, and succeeded in improving its food function-ality. This chapter reviews the enhancing effect of glycosylation through the Maillard reaction on food functionality of fis muscle protein and provides a discussion on the feasibility of using the protein glycosylation technology in the seafood industry.

30.2 Reaction between fish meat protein and reducing sugars through the Maillard reaction

Glycosylation using the Maillard reaction was performed with a modificatio for application to fis muscular proteins. Various kinds of reducing sugars (from monosaccharide to polysaccharide) can be attached to the protein molecules through the Maillard reaction. Briefl , fis meat proteins were suspended in 10 to 50 mM NaCl, mixed with reducing sugars, and then lyophilised. The lyophilised protein-sugar mixtures were incubated at 30 to 60°C under controlled relative humidity using a temperature- and humidity-controlled cabinet.

Figure 30.1 shows the effect of temperature on the reaction between the lysine residues of shellfis (scallop) Mf and glucose [13]. When lyophilised Mf with glucose was incubated at 40 to 60°C and 35% relative humidity (RH), the available lysine content decreased, and fructosamine, which is the Amadori rearrangement product in the early stage of the amino-carbonyl reaction, was produced simultaneously at all temperatures. These changes occurred rapidly with an increase in the reaction temperature. On the other hand, when the lyophilised Mf with sorbitol was incubated under the same conditions, the available
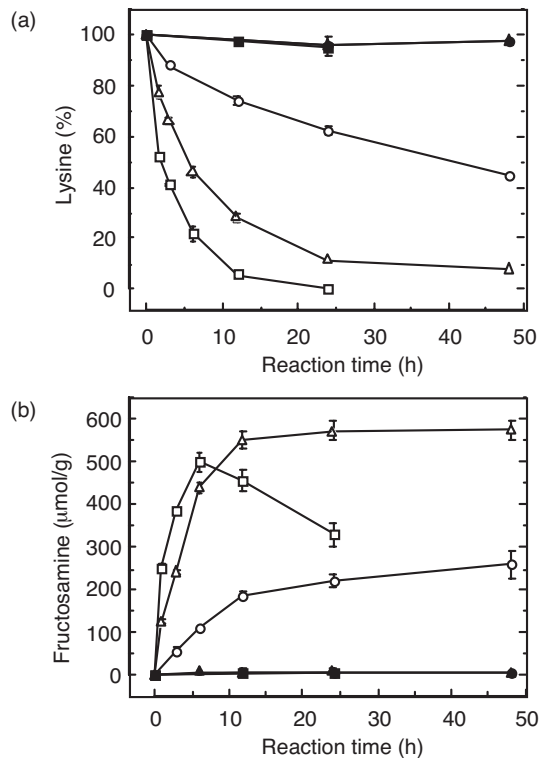
Fig. 30.1 Changes in the available (a) lysine and (b) fructosamine contents of scallop myofibrillar protein (Mf) during the reaction with glucose at different temperatures. Mf mixed with glucose (open circle, open triangle, and open quadrangle) or sorbitol (closed circle, closed triangle, and closed quadrangle) was lyophilised and incubated at 40°C (open circle and closed circle), 50°C (open triangle and closed triangle), and 60°C (open quadrangle and closed quadrangle) and RH 35%. Adapted with permission from Katayama *et al.* [13]. Copyright 2002, American Chemical Society.

lysine content remained unchanged, and no production of fructosamine was observed. These results indicate that the lysine residues in Mf were reacted with glucose through the Maillard reaction. It was previously reported that a polymerisation of Mf occurred in bovine [17] and squid [41] meats with the progress of the Maillard reaction with glucose. However, no polymerisation and no brown discoloration were observed in the glycosylation system [32]. These results indicate that the glycosylation was performed with regulating at the early stage of the Maillard reaction.

30.3 Suppression of protein denaturation at the Maillard reaction by controlling the reaction humidity

Humidity is an important factor affecting the Maillard reaction [15,44], as is the temperature [37]. For example, the Maillard reaction between the scallop Mf and glucose at 50°C progressed effectively under RH 35 to 65% [12]. In the case of the reaction between fis (chum salmon) Mf and alginate oligosaccharide (AO) at 60°C, the amount of AO bound to the protein increased with the rise in the RH of the reaction atmosphere. These results clearly indicate that moisture is an accelerating factor of the protein glycosylation. However, fis Mf denatures easily under high humidity, which impairs the protein functionality, and the improving effect of the glycosylation on the protein functionality (described below) is diminished as the protein denaturation progresses during glycosylation. Fortunately, some reducing sugars effectively suppress protein denaturation during lyophilisation, freezing, and heating [22,27]. For example, the loss in the solubility in 0.5 M NaCl and the Ca-ATPase activity of myosin in Mf were substantially reduced by the addition of more than 0.3 M glucose [32] or 2% (w/v) dextran [7] to the protein solution. Thus, the glycosylation of fis muscle protein should take place in relatively low humidity and in the presence of sorbitol or a large quantity of reducing sugars to suppress protein denaturation during glycosylation.

30.4 Water solubilisation of fish Mf protein by glycosylation

The major components of Mf, myosin, and actin, are insoluble in water and low-ionic-strength media, such as a physiological salt solution. For the high utilisation of protein waste and meat that is decreasing in freshness in the food industry, attempts have been made to solubilise fis meat in water using proteolytic enzymes and acid hydrolysis for use as human food, animal feed, and liquid fertiliser [1,24,28,29,38]. However, the production of peptides by protein hydrolysis is often responsible for a bitter and unacceptable taste. On the contrary, the solubility in water (in a low-ionic-strength medium) of fis myofibrilla protein is effectively improved by glycosylation without protein degradation [31].

   Figure 30.2 shows the NaCl concentration dependence of the solubility of fis (carp) Mf reacted with glucose. The solubility of unglycosylated myofibrilla protein in 0.01 to 0.1 M NaCl solutions was less than 14%, and a marked increase in the solubility was observed in the range of 0.16 to 0.3 M NaCl. On the other hand, the solubility of the glycosylated Mf (reaction time: 12 and 24 hours) increased remarkably in the range of 0.01 to 0.1 M NaCl. When 17% of the lysine residue was reacted with glucose, the solubility in 0.16 M NaCl
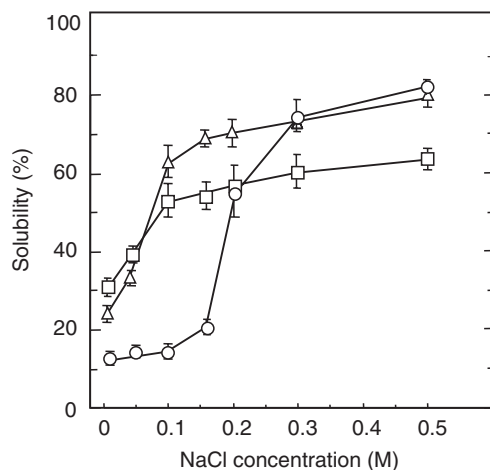
Fig. 30.2   NaCl concentration dependence of the solubility of carp myofibrillar protein reacted with glucose. Proteins were reacted with glucose at 40°C for 0 hour (circle), 12 hours (triangle), and 24 hours (quadrangle). Adapted with permission from Saeki & Inoue [30]. Copyright 1997, American Chemical Society.

reached 70%. Such an effect of the glycosylation on protein solubility was also observed when the Mf was reacted with ribose [31], maltose [11], and oligosaccharide [19].

The improved solubility of fis myofibrilla protein under physiological condition was impaired with the progress of the Maillard reaction [30,35]. In addition, the denatured myofibrilla protein could not be water-solubilised even if protein glycosylation progressed. Therefore, the simultaneous regulation of the Maillard reaction at an early stage and suppression of protein denaturation are important to achieve highly improved solubility of the protein [40]. A protectant having no reducing end carbonyl group, such as sorbitol, is useful to avoid protein denaturation during glycosylation [34,35].

30.5   Molecular mechanism of water solubilisation by glycosylation

The myosin molecule consists of two subfragments, the globular head portion (water-soluble), called subfragment-1 (S-1), and the water-insoluble (salt-soluble) long tail portion, called "rod" [43]. The rod region of myosin plays a significan role in the formation of the myosin filamen [10], and myosin molecules aggregate and assemble into insoluble filament when they exist in a low-ionic-strength medium and at neutral pH.

Figure 30.3 shows the solubility change in myosin and its subfragments as a result of glycosylation [12]. The solubility of carp and scallop myosin in 0.1 M NaCl increased markedly with the reaction time. The improved solubility became almost equal to the solubility in 0.5 M NaCl when about 60% of the available lysine was reacted with glucose. The solubility of S-1 in 0.1 M NaCl remained at a high level ($>$90%), regardless of the reaction with glucose. On the other hand, the solubility of the myosin rod in 0.1 M NaCl increased markedly and reached the same value as the solubility in 0.5 M NaCl. The results in Figure 30.3 indicate that the water solubilisation of myosin reflect the functional change of the rod region. The
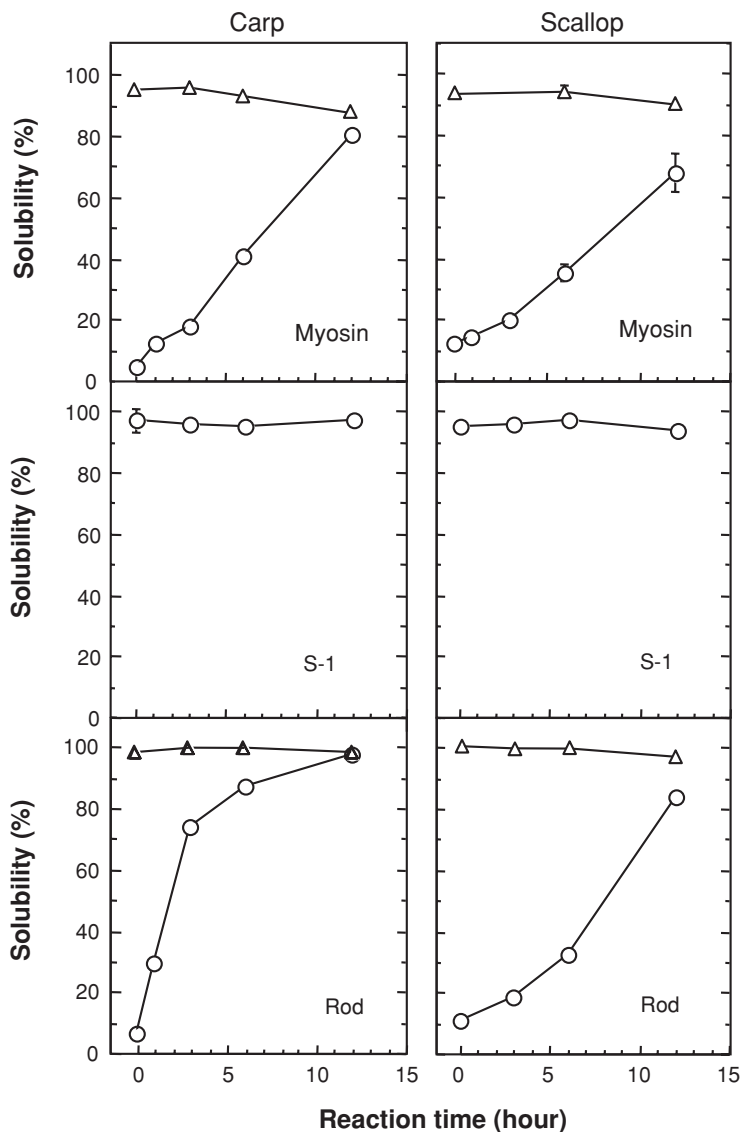
Fig. 30.3 Solubility changes in carp and scallop myosin and its subfragments during a reaction with glucose. Myosin, S-1, and rod reacted with glucose at 50°C were dissolved in 0.1 M (open circle) and 0.5 M (open triangle) NaCl (pH 7.5). Adapted with permission from Katayama *et al.* [12]. Copyright 2004, with permission from Elsevier.

water-soluble glycosylated myosin rods lost the filament-formin ability [12] and existed as a monomeric state in a low-ionic-strength medium [40]. The solubility characteristics of myosin are reflecte in the ionic-strength dependence of the solubility of the rod region, and the self-assembly of the myosin rod is accelerated by a decrease in the negative charge repulsion [23]. Therefore, water solubilisation of myosin molecules is attributed to the loss of positively charged lysine residues (ε-amino groups) by the glycosylation.
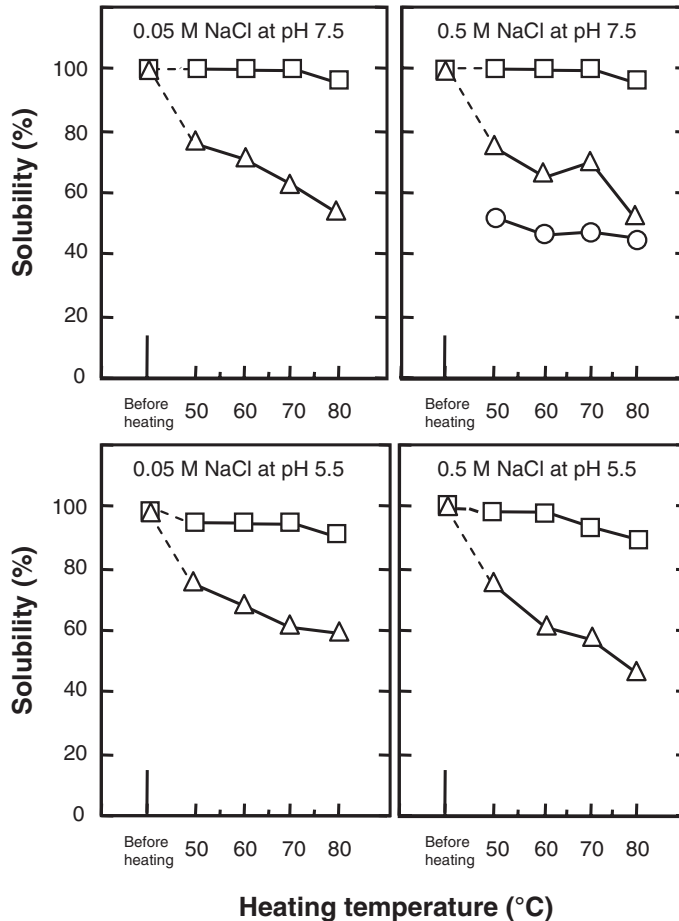
Fig. 30.4 Effect of heating at different NaCl concentrations and pH levels on the solubility of Mf-AO conjugate. Native Mf (circle) and Mf-AOs containing 120 µg/mg (triangle) and 227 µg/mg (square) of AO were dissolved in 0.05 M or 0.5 M NaCl (pH 5.5 and 7.5 in both cases) and heated at 50–80°C for 2 hours. Adapted with permission from Sato *et al.* [34]. Copyright 2003, American Chemical Society.

30.6   Improvement of the thermal stability and emulsion-forming ability of fish myofibrillar protein

There are some reports in which the glycosylation enhances the thermal stability of food proteins [2,6,16,26,42], and the same functional improvement was also observed in fis Mf [7,19]. Figure 30.4 shows the thermal stability of carp Mf conjugated with alginate oligosaccharide (Mf-AO conjugate) under different NaCl concentrations and pH levels [34]. Mf-AO conjugates have higher stability in a wide range of NaCl concentrations, pH levels, and thermal conditions than native Mf. The solubility of native Mf dissolved in 0.5 M NaCl (pH 7.5) decreased to around 50% of its original solubility when heated at 50–80°C for 2 hours. The solubility of a heat-treated Mf-AO conjugate containing 120 µg/mg of AO in 0.05 and 0.5 M NaCl (pH 7.5) was significantl higher than that of native Mf, although the solubility tended to diminish as the heating temperature increased. Furthermore, the heat

treatment had no effect on the solubility of an Mf-AO conjugate containing 227 µg/mg of AO regardless of the heating temperature or NaCl concentration. Improved thermal stability of Mf by conjugation with AO was also observed at pH 5.5, in which the native Mf was insoluble at both NaCl concentrations. The emulsion-forming ability is one of the important characteristics of food protein [20,25]. The emulsion-forming of fis Mf was effectively improved by conjugation with oligo- and polysaccharides [7,34]. In addition, the improved emulsion-forming ability of carp Mf-AO conjugate remained unchanged after heated at 80°C for 2 hours in 0.05 to 0.5 M NaCl and pH 5.5 to 7.5 [34]. Nakamura *et al.* [26] suggested that emulsion stability enhanced by glycosylated proteins is closely related to the polysaccharide chains attached to the protein. In other words, the glycosylated proteins are absorbed at the surface of oil droplets, and polysaccharide chains attached to the protein suppress the association of each oil droplet. The polysaccharide chains attached to the protein may also inhibit aggregation among protein molecules during the heating of the protein solution.

30.7 Complex utilisation of under-utilised marine bioresources using the glycosylation system

In this chapter, a complex glycosylation system for using under-utilised marine bioresources is proposed for developing functional fis protein material. This is a new scheme using glycosylation technology and some kinds of fis waste, and under-utilised resources are cooperatively utilised through this system. Figure 30.5 shows a scheme for the complex utilisation of little-utilised marine bioresources to develop a fis meat protein with excellent food functionalities. Three kinds of under-utilised resources were employed in this system:

1) spawned-out salmon as a protein resource;
2) under-utilised brown algae as a material of alginate;
3) a specifi enzyme from marine bacteria.

Spawned-out salmon decreases in quality as a food material (texture, taste, and fl vour) and generates a bad smell as a result of the severe stress caused by spawning. Therefore, a
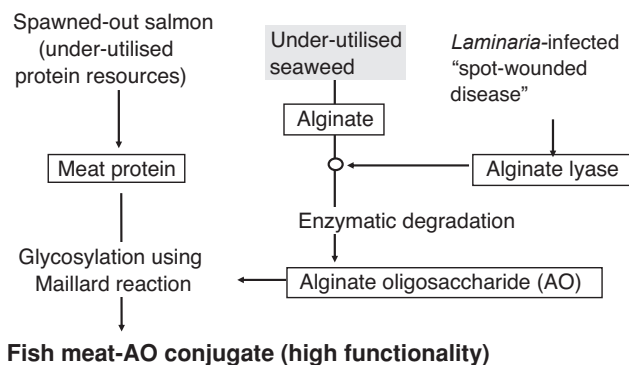
Fig. 30.5 Outline of the complex utilisation of under-utilised marine bioresources to prepare new functional fish meat protein.

significan percentage of salmon meat is discarded and burned as an industrial waste after the roe has been collected. However, the discarded fis meat is still a valuable protein resource. Therefore, we attempted to develop a new food material from mature salmon meat protein using the potential of other under-utilised resources. The following protocol was used:

1) under-utilised seaweed, brown algae, was used as the alginate material;
2) a marine bacterium, *Pseudoalteromonous elyacovi*, which is the pathogen of the spot-wound disease of Laminaria, was used to originate alginate lyase;
3) AO was prepared from the alginate of under-utilised algae using the alginate lyase; and
4) salmon meat-AO conjugate was prepared through the Maillard reaction.

In this scheme, water-washed meat was used as a raw material, and the water-soluble and thermally stable fis protein was produced in a stable form by the Maillard reaction with AO. This suggests that the method investigated here has strong potential for use with spawned-out chum salmon.

30.8   Food safety check of fish meat protein conjugated with AO

The food safety of the protein-sugar conjugate was assessed *in vitro* and *in vivo* [33,39]. The results were as follows.

1) No mutagenicity was observed in the Rec assay and the Ames test at 10 mg/mL of meat-AO conjugate (AO contained 120 μg/mg of protein).
2) No toxicity was observed in the acute toxicity test by oral administration of 2,000 mg/kg weight of meat-AO conjugate to Sprague-Dawley (SD) rats.
3) When 500 mg/kg weight of meat-AO conjugate was orally administered to SD rats over a 4-week period for a semi-acute toxicity test, it was confirme   that meat-AO had no effect on rat growth.

These results clearly indicate that meat-AO conjugate (Fig. 30.5) had no toxicity and presented no food safety problems.

30.9   Conclusions

Protein glycosylation using the Maillard reaction is superior to other types of chemical mod-ificatio for food proteins, because it proceeds under mild and safe conditions without any chemicals. The enhancing effect of the glycosylation changes depends on the sugars attached to the protein; The solubility of fis meat protein in a low-ionic-strength medium was highly improved by the reaction with low-molecular-weight sugars, and some reducing sugars im-proved the protein stability. In addition, glycosylation with oligo- and polysaccharides helped to improve the emulsion-forming ability. These finding may contribute to the development of new systems for higher utilisation of marine bioresources in the seafood industry.

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31 Value addition to seafood processing discards

Sachindra M. Nakkarike, Bhaskar Narayan, Masashi Hosokawa, and Kazuo Miyashita

31.1   Introduction

Fish processing operations generate large quantities of discards in the form of inedible by-products, which are highly perishable and dumping of these by-products results not only in the loss of huge amounts of protein rich material but also lead to pollution problems. The solid waste management in fis processing industries includes their recycling into marketable products. These by-products are an important source of a variety of bioactive materials. Discards from seafood processing plants have been reported to contain various biomolecules such as valuable/specialty enzymes, pigments like carotenoids, collagen, gelatin, bioactive peptides, and polyunsaturated fatty acids (PUFA) rich lipids, etc. Hence it is appropriate that the discards from the fis processing industry should be looked upon as a valuable raw material for the recovery of bioactive compounds. If these biological compounds can be recovered, they will serve the dual purpose of recovery of these biomolecules and reduce the pollution problems associated therewith. An attempt is made to review the products of commercial utility that are derived from by-products and value added bioactive molecules that can be derived from fis processing discards. This chapter summarizes the research efforts on recovery of valuable biocomponents such as enzymes and protein hydrolysates, including bioactive peptides, collagen, chitin/chitosan, and carotenoids from seafood by-products.

31.2   Enzymes from seafood discards

Fish visceral discards generally contains high quantities of digestive enzymes, such as collagenases, trypsin, chymotrypsin, elastase, and carboxypeptidase that exhibit interesting properties, implying that this material has a potential for biotechnological utilization [1]. As the marine organisms adapt to different environmental conditions, their porteinases are reported to have better properties, such as higher catalytic activity at low temperatures and at broader pH ranges [2].

There are various methods for enzyme recovery, such as ammonium sulphate precipitation and dialysis, through ensiling and ohmic heating. A general procedure for the extraction of acid and alkaline proteases from fis viscera is given in Fig. 31.1. Ensiling of biological
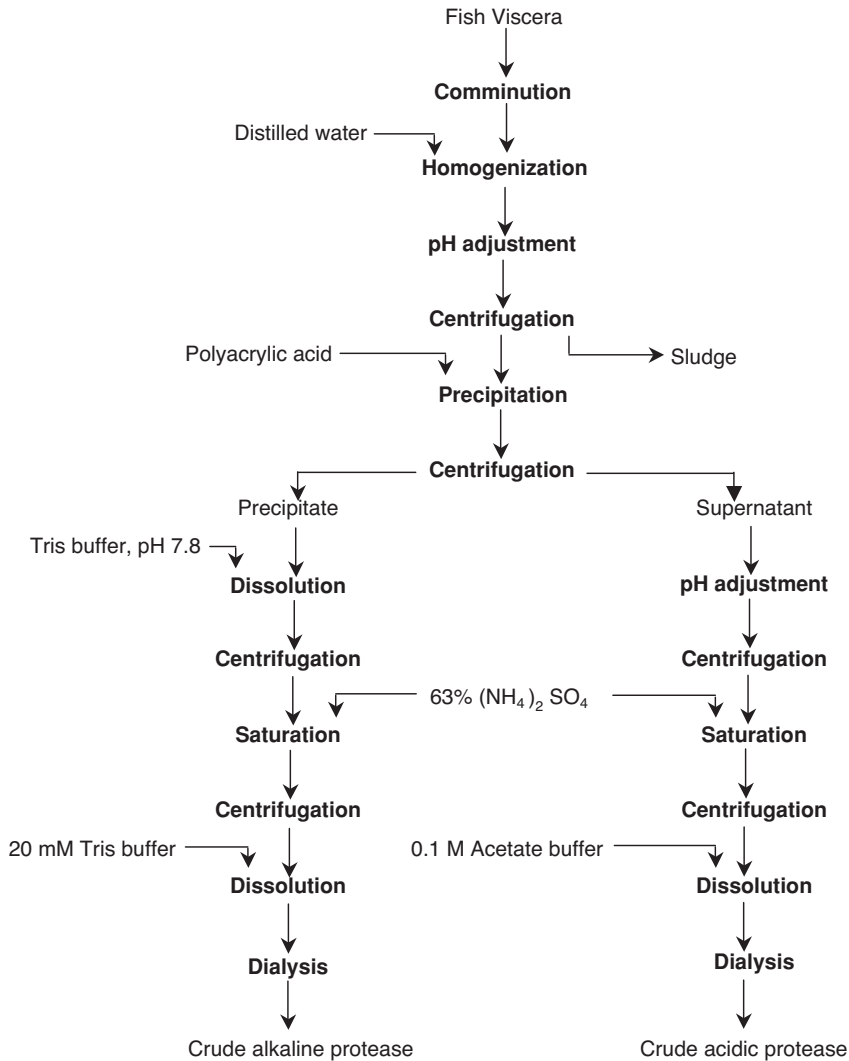
Fig. 31.1 Extraction method for acid and alkaline proteases from fish viscera. Adapted from Shahidi & Kamil [1]. Copyright 2001, with permission from Elsevier.

materials is performed to stabilize them against deterioration. However, it has been shown that the activity of proteases found in the visceral wastes of freshwater carps decreases with storage in the ensiled form [3]. Ohmic heating process was successfully applied to recover heat stable cathepsin from surimi wash water. The recovery of enzyme can be facilitated by other techniques such as precipitation, chromatography, and two-phase aqueous systems [4].

In addition to proteases, the enzymes that have been isolated from fis and shellfis discards include chitinases, lipases, and trasglutaminase [1]. Chitinases from marine source have been isolated from digestive organs of fis and crustacean wastes [5]. Shrimp processing waste was attempted as a substrate for production of microbial chitinases by solid-state fermentation [6].

Seafoods are considered as an important source of lipases. Isolation and characterization of lipases from different marine sources have been documented [1]. The activity of lipases with respect to catalyzing esterification hydrolysis, or exchange of fatty acids in esters provides an opportunity for producing new types of triacylglycerols (TAG), esters, and fatty acids [7]. This property of lipases has been used in production of TAG enriched with omega-3 (n-3 or ω-3) PUFA from fis oil [8].

Enzymes from marine resources have various industrial applications. Shahidi and Kamil [1] have summarized the various potential applications of marine enzymes. The potential applications include deskinning of fis and squid, purificatio and production of fis roe, descaling and production of pearl essence, removal of organs and membranes from seafood products, extraction of carotenoproteins from crustacean wastes, fermentation of fish pro- duction of fis sauce, fis silage, production of protein hydrolysate, production of chitin and related products, and PUFA enrichment of fis oils, etc.

31.3 Protein hydrolysate and bioactive peptides from seafood discards

Fish protein hydrolysate can be prepared from cheap pelagic fish by-products from the fil leting industry, by catch from trawlers, and fis processing discards. Hydrolysis of proteins improves the solubility that depends on functional properties of fis proteins [9]. Hydrol- ysis of fis proteins can be achieved by both the digestive enzymes of fis itself, as well as by addition of external sources of enzymes [10]. The method of production of fis pro- tein hydrolysate involves mixing the ground fis with endogenous enzymes or an external source of protease and incubation in a reactor under optimum conditions for enzyme activity [11]. The enzyme needs to be inactivated by heating to stop the hydrolysis reaction after a specifi time interval. The hydrolyzed liquor is then separated by sieving and centrifugation, and concentrated by spray drying. The common procedure for preparation of fis protein hydrolysate is outlined in Fig. 31.2 [12].

Even though hydrolysis of fis proteins improves the functionality, the properties of the product depend on the choice of enzymes and process conditions [13]. The solubility of fis proteins increases with increase in the degree of hydrolysis (DH), which depends on the incubation time and the amount of enzyme added [14]. Better emulsifying capacity and emulsion stability, and foaming properties of protein hydrolysate are observed at a low DH [15]. Prolonged hydrolysis results in the production of short chain peptides, lacking the functional properties of native proteins [16]. The most common commercial proteases used for preparation of fis protein hydrolysate include those from plant sources such as papain or from animal origin such as pepsin, chymotrypsin, or trypsin [17]. Microbial enzymes have also been applied for the hydrolysis of fish which offer several advantages, including a wide variety of available catalytic activities and greater pH and temperature stabilities [18].

The hydrolysates have a wide range of potential applications as they may be used as fl vour enhancers, functional ingredients, or as nutritional additives to foods of low protein quality. Fish protein hydrolysate can be employed as "milk replacers" for young calves, which will eventually be reared for milk or beef production [12]. One of the important uses of protein hydrolysate is as the base material for isolation of bioactive peptides [17]. Peptides isolated from hydrolysates of marine raw materials have been demonstrated to possess several bioactive properties, such as inhibition of angiotensin I converting enzyme (ACE) activity

Fish
|
Mincing

Water ———————→|←——————— Enzyme
Hydrolysis

(Moderate heating and stirring
|
Sieving ———→ Bones and scales
|
Solids ←——— **Centrifugation** ——→ Oil
|
Evaporation
|
Spray drying
↓
Soluble fish protein hydrolysate
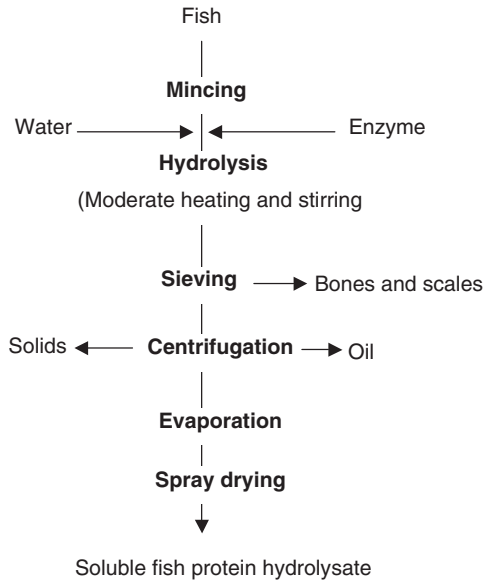
Fig. 31.2   Common procedure for fish protein hydrolysate production. Adapted with permission from Gildberg [12]. Copyright 1993, with permission from Elsevier.

and lowering of blood pressure. The mechanism of inhibition of ACE activity was found to be dependent on amino acid pairs and the amino acid sequence [19]. Even the collagen peptides isolated from hydrolysates of fis skin and scales were found to possess high ACE inhibitory activity [20].

Protein hydrolysates from different marine proteins have been found to possess antioxidant activity [21–23]. The antioxidant activities of protein hydrolysates are reported to be determined by the level and composition of free amino acids and peptides [24]. Antioxidative activity of protein hydrolysate from marine resources varied with DH, enzyme used, and the body part from which the protein hydrolysate is prepared [22,25]. Protein powders prepared from fermented shrimp waste also exhibits high antioxidant activity [26].

31.4   Collagen and gelatin from fish discards

Collagen, the major structural component of skin, bone, tendon, and cartilage is one of the most abundant proteins in vertebrates and constitutes about 25% of total proteins. Denaturation of collagen yields gelatin. Collagen find applications in the cosmetics, biomedical, and pharmaceutical industries, while gelatin find several applications in food and biomedical industries. Traditionally, collagen is derived from land-based animals, mainly from porcine and bovine sources. However, with the outbreak of bovine spongiform encephalopathy (BSE) and foot-and-mouth disease (FMD), and due to aesthetic and religious reasons, the use of collagen and gelatin from land-based animals is becoming restricted [27].

Seafood processing by-products are one of the most important sources of collagen, which can be isolated from fis skin, bones, and fin of fis processing discards [17]. Collagen from different marine species has been characterized [28–30]. Collagen is generally extracted

and solubilized by acid treatment of the material without altering the triple helix structure [17]. Sadowska *et al.* [31] established the optimum conditions for the extraction of collagen from skins of cod using citric acid. Enzymatic digestion has been suggested as a method for isolation of pepsin-soluble collagen from fis discards [28]. Thermal treatment of collagen alters the triple helix structure by cleavage of the hydrogen and covalent bonds, forming coiled structure of gelatine [32]. This characteristic of collagen is used to extract gelatin by solubilizing the collagen by hot water treatment. In the process detailed by Arnesen and Gildberg [33], gelatin from cod head and salmon skin was extracted under acidic conditions at elevated temperatures, after initial removal of other muscle proteins by successive alkaline and acid extractions.

The use of gelatin in the food industry is mainly to improve the texture, water holding capacity, and stability of food products [34]. Gelling properties of gelatin are important criteria for its use in the food industry. Fish gelatin is known to be inferior to conventional gelatin from land animals due to its lower melting and gelling points [35] and weaker gel strength [36]. The lower gelling strength of fis gelatin was utilized towards industrial applications such as micro-encapsulation, light sensitive coatings, low-set time glues, and cosmetics [10]. The biomedical application of collagen/gelatin is mainly as carriers for drugs [37]. Collagen film are attempted as drug carriers for the treatment of cancer [38] and also as gene delivery agents promoting bone and cartilage formation [39].

31.5 Chitin and chitosan from crustacean discards

Chitin is the secondmost abundant natural polysaccharide in Nature, next only to cellulose. Solid discards from processing of crustaceans provide an important source for industrial production of chitin. Chitin can prepared from the shells of crustaceans such as shrimp and crab [40,41]. The chitin content in the crustacean exoskeleton ranges from 13 to 42%, depending on the species [42]. The process of chitin preparation from shellfis discards involves deprotenization, demineralization, and decolourization. Deprotenization of ground shell mass is carried out by treating with alkali (sodium hydroxide ranging from 1–10%) at elevated temperatures of 65 to 100°C [43]. Demineralization is carried out by treating the deprotenized shells with 1 to 8% hydrochloric acid at room temperatures for 1 to 3 hours [44]. Decolourization of the resultant chitin is done by treating with organic solvents, either hydrogen peroxide or sodium hypochlorite [45].

The traditional process of deprotenization uses strong alkali, higher temperature, and prolonged treatment period. The prolonged alkaline treatment results in depolymerization and deacetylation of chitin [43]. Alternative methods using milder treatments such as use of enzymes and fermentation have been attempted. These alternative processes for chitin production allow for the recovery of other valuable products such as protein and carotenoids [46]. Synowiecki and Al-Khateeb [43] proposed an enzymatic method for deprotenization of crustacean shell and recovery of protein along with chitin. Protease producing micro-organisms have been successfully used for deprotenization of crustacean discards [47].

Fermentation of crustacean processing discards using lactic acid bacteria has been found to be effective in deprotenization and demineralization. Fermentation of shrimp waste with *Lactobacillus plantarum* results in up to 80% deprotenization and demineralization [48]. In a later study by Jung *et al.* [49], co-fermentation of red crab shell with an acid producing bacterium (*Lactobacillus paracasei*) and a proteolytic bacterium (*Serratia marcescens*) resulted in an efficien mineralization and deprotenization. Fermentation of shrimp waste under

optimized conditions using *Pediococcus acidolactici* resulted in deproteinization close to 98% and demineralization close to 73% within 72 hours of fermentation [50].

Chitosan is produced by deacetylation of chitin by treatment in concentrated alkaline solution at very high temperatures [51]. The properties of resultant chitosan depend on the treatment conditions such as concentration of alkali, time, and temperature or process. Decrease in the viscosity and molecular weight of chitosan can be avoided by elimination of oxygen during deacetylation, by processing under nitrogen [52], or with the use of oxygen scavengers [53]. To avoid the use of strong alkali in production of chitosan, use of chitin deacetylase derived from fungi has been attempted [54].

Solubility problems associated with chitin/chitosan has resulted in development of methods for preparation of water soluble oligomers of chitin and chitosan [41,55]. Oligomers can be prepared by acid hydrolysis as well as by enzymatic approaches. The enzymatic method is preferred over acid hydrolysis due to the reason of higher yield and higher degree of polymerization in enzymatic hydrolysis [56]. Oligomers of chitin and chitosan are known to possess several beneficia functions such as antitumor, antioxidant, and ACE inhibitory activity [17].

The industrial application potential of chitin and its derivatives is widespread for textile, cosmetics, pharmaceuticals, and food [45]. Chitin and its derivatives, being non-toxic, fin several biomedical applications such as antiviral, antitumour, fungistatic, and bacteriostatic agents [51]. Chitosan has the potential application in preparation of superabsorbant material for body fluid in disposable medical and personal care products [57]. The application of chitin/chitosan in the food industry is as antimicrobial agent, edible packaging films additive in various food preparations, nutritional enhancer, and an agent for recovery of products from processing waste [45].

31.6 Carotenoids from crustacean discards

Discards from processing of crustaceans such as shrimp, crabs, and krill are one of the important natural sources of carotenoids, particularly astaxanthin [58–61]. Several studies have been carried out to recover the pigment from crustacean processing discards. Methods such as extraction of carotenoids using organic solvents and edible oils and recovery of carotenoids as carotenoprotein are available. Carotenoids in shrimp waste can be extracted using cold acetone and subsequently partitioned using petroleum ether [62]. The supercritical CO_2 method with ethanol as cosolvent has also been attempted for astaxanthin extraction from crawfis shells [63]. A mixture of polar and non-polar solvents for improved recovery of carotenoids from shrimp waste has been demonstrated [64] and the process was optimized, as shown in Fig. 31.3 [65].

As carotenoids in crustacean wastes are fat soluble, vegetable oils have been used to extract pigments from waste. Chen and Meyers [66] used enzymatic hydrolysis of homogenized crawfis waste with a protease and subsequent extraction with soybean oil for recovery of carotenoids. In a patented process for utilization of crustacean shell waste [67], the crawfis waste was homogenized, acidified and heated with soybean oil to recover the pigment-enriched oil. The extraction of carotenoids using different oils such as soybean, cottonseed, herring, menhaden, and salmon oil was attempted by Chen and Meyers [68]. No and Meyers [69] demonstrated that the process of oil extraction of carotenoids from crawfis waste can be integrated with production of chitin and chitosan. Use of sunfl wer oil was found to give higher carotenoid yield and the extraction conditions were optimized [70]. The oil extraction process of shrimp waste carotenoids is presented in Fig. 31.4 [65]. A method has been

Shrimp waste

|

Communition

Isopropyl alcohol:hexane (40:60)* ⟶ |

Homogenization   ⟵

2 times

Centrifugation

——————— Residue

Supernatant

|

Washing with saline

|

Phase separation

⟶ Saline + IPA

Hexane layer

|

Concentration under vacuum

↓ ——————— ⟶ Recovered hexane

Carotenoid concentrate

* Solvent mixture to waste ratio of 5:1
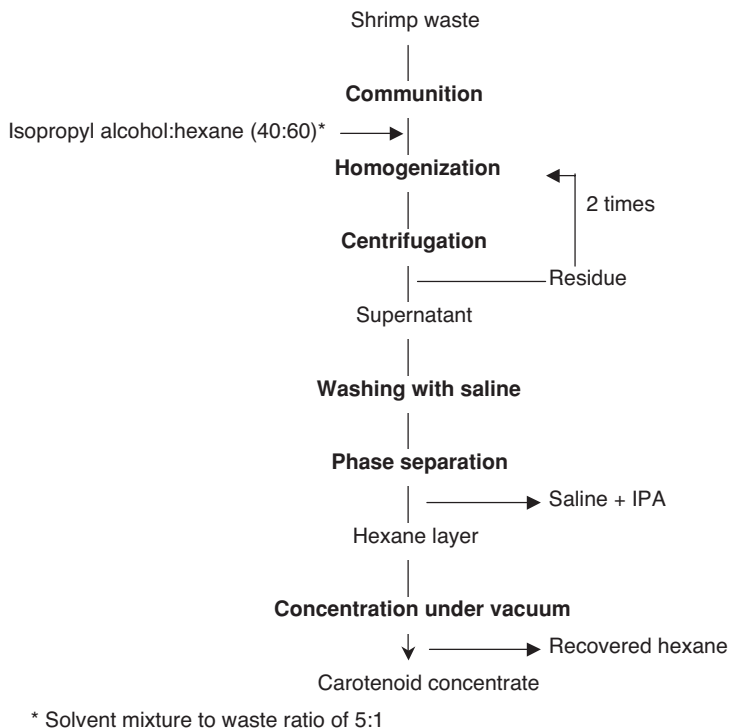
Fig. 31.3   Process for extraction shrimp waste carotenoids using organic solvents. Adapted from Sachindra [65].

Shrimp waste

|

Communition

Vegetable oil* ⟶

Mixing

|

Heating (at 70°C for 150 min)

|

Centrifugation

|

Phase separation

↓ ⟶ Residue

Pigmented oil

*Vegetable oil to waste ratio of 2:1 (v/w)
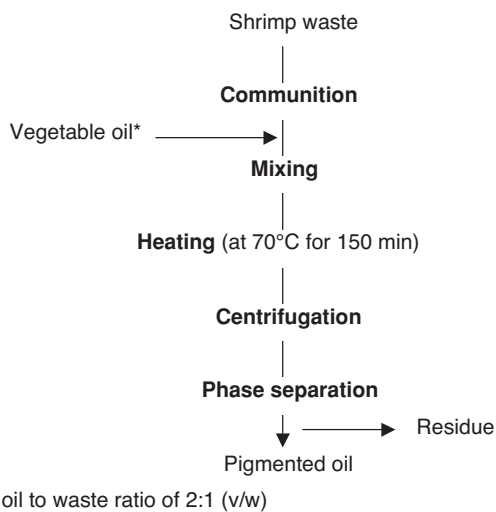
Fig. 31.4   Process for extraction shrimp waste carotenoids using vegetable oil. Adapted from Sachindra [65].

developed based on silica gel column chromatography for concentration of carotenoids in krill oil [71]. Conditions for supercritical CO_2 extraction of astaxanthin from crab shell waste using ethanol as cosolvent has been standardized by Felix-Valenzuela *et al*. [72].

Torrison *et al*. [73] attempted acid ensilaging as a method for stabilization of astaxanthin in shrimp waste during storage prior to oil extraction and acid ensiling of crawfis waste was found to stabilize the astaxanthin in the waste and also increased the recovery of astaxanthin in soybean oil [74]. The crude oil extract from shrimp waste silage was found to be more concentrated in astaxanthin than the oil obtained from raw shrimp waste [75]. Guillou *et al*. [76] observed that ensiling of shrimp waste was effective in stabilizing astaxanthin in the waste and also increasing the yield of carotenoid recovery by solvent extraction. In a study that comparatively evaluated the effect of acid and fermentation ensiling on the stability of shrimp waste carotenoids [77], fermentation ensiling was found to be superior to acid ensiling in terms of carotenoid recovery from the ensiled mass, both in solvents and oil.

As carotenoids are more stable as a complex with proteins, studies have been carried out on recovery of carotenoids as carotenoproteins. Simpson and Haard [78] developed an enzymatic technique for extraction of carotenoprotein from shrimp waste using chelating agents such as ethylenediaminetetraacetic acid (EDTA) and proteolytic enzymes such as trypsin. Cano-Lopez *et al*. [79] used trypsin from Atlantic cod instead of bovine trypsin for increased recovery of carotenoprotein from shrimp waste. Carotenoprotein from crawfis waste has also been extracted by a fermentation process [80].

Carotenoids extracted from crustacean discards may fin potential use as a source of pigmentation in cultured fis and shrimp. Aquaculture has become one of the major practices for continuous supply of aquatic animals. Aquatic animals grown in the wild depend on their diet for carotenoid requirements. Aquatic animals, which are cultured, do not show the same coloration as that of their wild counterparts [81]. Pigmentation of cultured species, such as salmonids and crustacean, is done through dietary manipulation [58]. Both synthetic carotenoids and natural pigment sources have been used for pigmentation of cultured fish Synthetic astaxanthin and canthaxanthin. either alone or in combination. are most commonly used for pigmentation of salmonids [82]. However, it has been noted that synthetic canthaxanthin produces a yellow-orange colour, not the natural colour of wild grown salmon [83]. However, the use of synthetic pigments in aquaculture is not favoured in many countries. In the EU countries, the presence of canthaxanthin in smoked fis fillet is prohibited [84].

The best alternative to synthetic carotenoids would be the use of natural carotenoids for fis pigmentation. Pigmentation of cultured salmonids has been achieved with inclusion of crustacean waste in their diets [85]. The use of crustacean meals as a pigment source in feed is not desired because of low carotenoid content and high calcium and chitin level. Thus, attempts have been made to use concentrated carotenoid extracts from crustacean waste. The role of carotenoids in aquaculture has been summarized by Shahidi *et al*. [58].

31.7   Conclusions

Judicious waste management technique is an important criterion for the success of any food processing industry. Fish processing discards, being highly perishable, require special attention. With the normal disposal of seafood processing discards being objected to by the pollution monitoring authorities and the general public, attempts for their conversion to value added marketable products is gaining importance. With the ever expanding aquaculture industry worldwide, large quantities of material will be available for processing, thus increasing

the waste load. Recovery of bioactive components such as enzymes, proteins, and pigments is the appropriate solution to the problem of waste disposal in the fisher industry. The efficien utilization of fis processing discards for the recovery of value added by-products is essential and desirable as an environmentally friendly approach, to minimize the safety risks associated with waste. This will not only reduce the pollution problems but also improve the economics of processing. It is also essential that efforts be made for total utilization of processing discards by adopting an integrated approach for the recovery of all the valuable components in a single operation, thereby reducing the cost of recovery and avoiding the problem of disposal of residual materials after recovery of components. Furthermore, it is important to adopt mild treatments such as use of enzymes or biological techniques (e.g. fermentation) to avoid the use of chemicals.

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32 Role of marine foods in prevention of obesity

Shigeru Nakajima

32.1 Introduction

Marine foods often attract the attention of researchers because of the health benefit they offer. Health benefit of foods are mainly attributed to their lipids, especially fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and proteins mainly due to higher content of important amino acids such as lysine and histidine. Although the entire molecular mechanism has not been clearly elucidated, data accumulated from experimental animal and human studies supports the beneficia role of dietary marine compounds in obesity therapy.

Obesity is now recognized as a secondmost important preventable cause of death, exceeded only by cigarette smoking. Obesity is a potent risk factor for type-2 diabetes, hypertension, and dyslipidemia, co-morbidities that markedly increase the risk of cardiovascular disease. Obesity is a multifunctional condition affected by the combined effects of genes, environment, and their interactions. Other important parameters considered to explain increase in obesity are food availability, increased dietary fat content, greater energy density of foods, and decreased physical activity. Thus, a large number of studies have investigated the role of food components for the prevention of obesity [1–5].

Recent increased interest on marine foods can mainly be attributed to the health benefit they offer to humans. Epidemiologic evidence strongly supports the important role of marine components for human health. Although lipid can be stored far more efficientl than other nutrients, data accumulated from experimental animal and human studies clearly support a beneficia role of dietary marine lipids in weight management. However, relatively little has been done to study the effects of other marine compounds on obesity therapy. In addition, the details in the anti-obesity mechanism for marine lipids have not been fully elucidated. In this chapter, an effort is made to focus on the anti-obesity effect of marine lipids and histidine from marine origins.

32.2   Anti-obesity effect of marine lipids

32.2.1   Molecular mechanism for anti-obesity effect of marine lipids

Over the centuries, epidemiological studies have revealed the lower incidence of cardiac related diseases in seafood eating populations. Furthermore, over the past several decades, different studies involving human and animal subjects have clarifie the biological activities of long-chain polyunsaturated fatty acids (PUFA) of marine origin, especially EPA and DHA. These studies have also resulted in both EPA and DHA becoming the firs marine nutraceuticals to be generally recognized as safe (GRAS) [6,7]. The health benefit of EPA and DHA are presented in Table 32.1. Most of these health beneficia effects play an important role in the prevention of obesity and obesity-related health complications.

Effect of EPA and DHA on lipid metabolism is strongly correlated to anti-obesity effects of marine lipids. PUFA intake may lower plasma LDL-cholesterol and triacylglycerols (TAGs) concentrations by inhibiting TAG and very low-density lipoproteins (VLDL) synthesis in the liver and by stimulating the synthesis of membrane phospholipids. This aids in preventing obesity-related problems. Some publications have described the reducing effect of fis oil on the abdominal fat pad [2,3]). Parrish *et al*. [2] reported that lard-fed rats had 77% more fat in perirenal fat pads and 51% more fat in epididymal fat pads compared with fis oil-fed rats.

Anti-obesity effect of fis oil was reported by Kawada *et al*. [8], who found that the expression of uncoupling protein (UCP1) in brown adipose tissue (BAT) was significantl higher in the fis oil diet-fed rats compared to that in the lard-fed group. In BAT mitochondria, substrate oxidation is poorly coupled to ATP synthesis because of the presence of UCP1, thereby leading to energy dissipation, that is, heat production. Kawada *et al*. [8] suggested that the intake of PUFA, such as EPA and DHA found in fis oil, causes UCP induction and enhancement of thermogenesis, resulting in suppression of the excessive growth of the abdominal fat pad. PUFA from vegetable oils also suppressed the excessive accumulation of adipose tissue, as compared to animal fats [9,10]. However, the activity of PUFA from vegetable oils was less than EPA and DHA from fis oil [8].

A great deal of interest has focused on adaptive thermogenesis by UCP families (UCP1, UCP2, and UCP3) as a physiological defence against obesity, hyperlipidemia, and dia-betes [11,12]. Involvement of BAT in cold-induced thermogenesis is well established and data from rodents have also demonstrated its role in diet-induced thermogenesis [13,14].

Table 32.1   Biological activities of EPA and DHA

Common biological activities of EPA and DHA
Antithrombotic effect
Hypolipidemic effect (lowering of blood lipids)
Increase in heart rate variability
Inhibitory effect on atherosclerosis
Anti-cancer activity
Anti-allergenic activity
Anti-inflammatory effect
Anti-diabetic effect

Biological activities of DHA
Mental health improvement
Retinal function improvement

Thermogenesis in BAT is due to UCP1, which is a dimeric protein present in the inner mitochondrial membrane of BAT, and it dissipates the pH-gradient generated by oxidative phospholyration, releasing chemical energy as heat. UCP1 is exclusively expressed in BAT, where the gene expression is increased by cold, adrenergic stimulation, β3-agonists, retinoids, and thyroid hormones [15]. Thermogenic activity of BAT is dependent on UCP1 expression level controlled by the sympathetic nervous system via noradrenaline [16–19]. As a consequence of noradrenaline binding to the adipocyte plasma membrane, protein kinase (PKA) is expressed, and then cyclic AMP response element binding protein (CREB) and hormone-sensitive lipase (HSL) are expressed. HSL stimulates lipolysis and the free fatty acids (FFA) liberated serve as a substrate in BAT thermognesis [19]. They also act as cytosolic second messengers, which activate UCP1 as PPARγ ligand. The same activity is expected in dietary polyunsaturated fatty acids [20]. The anti-obesity effect of dietary EPA and DHA may be partly due to their control of PPARγ expression [8]. DHA and EPA inhibit cyclocoxygenase, thereby reducing the amount of prostaglandins and increasing lipoxygenase activity. This, in turn, results in higher production of hydoxyeicosatrienoic acids (HETE) and leukotriene B4. Eicosanoids can act as transcriptional regulators of UCP. The anti-obesity effect of fis oil may, in part, be correlated with the regulatory effect of both EPA and DHA on eicosanoid formation.

32.2.2 Traditional marine products as a good source of anti-obesity PUFA, EPA, and DHA

The importance of omega-3 and -6 (n-3 and n-6) PUFA on human health has been demonstrated beyond any doubt through research work worldwide. They have several beneficia health and physiological effects. The functions of each n-3 or n-6 PUFA have attracted consumer attention and are often used in functional foods and nutraceuticals. EPA and DHA are the two PUFA found in marine lipids. These two long-chain PUFA have been shown to cause significan biochemical and physiological changes in the body [21–25], which mostly result in positive influence on human nutrition and health.

Although marine food consumption is very helpful in the prevention of lifestyle-related diseases, especially those related to obesity, consumption of EPA and DHA in unoxidized forms is very essential to the health benefit they offer. Both EPA and DHA, being highly unsaturated long-chain PUFA, are often prone to oxidation and then oxidized products cause undesirable fl vours and lower the nutritional quality and safety of lipid-containing foods. In addition, they may be potential carcinogens. Hence, oxidative deterioration of functional PUFA still remains the biggest problem in utilizing PUFA-rich oils in food applications. Owing to this, in all investigations on the dietary effects of these n-3 PUFA, lipid peroxidation has received considerable attention because of its possible contribution to the potential damage of biological systems [26–32].

Processing of marine foods and the cooking process involved during preparation of culinary items based on marine foods, generally, might result in degradation of lipids. Thus, it becomes clear that processing and cooking steps involved in preparation of marine foods could potentially result in oxidized lipids. On the contrary, the Japanese population has been consuming many types of processed marine foods traditionally. It has been reported that marine lipids are scarcely oxidized in such kinds of processed foods, even after heat treatment [33–35]. When quantitative changes in EPA and DHA are determined during the production of processed marine foods subjected to thermal processing, such as boiled fis paste (kamaboko) and dried marine foods (Himono), little changes have been observed

Raw fish

↓

Fillet

Water bleaching
Dehydration

↓

Minced meat

↓ Grinding

Grinded meat (Surimi)

Seasoning
Heating (180°C)
Cooling

↓

Fired Kamaboko (*Jyakoten*)
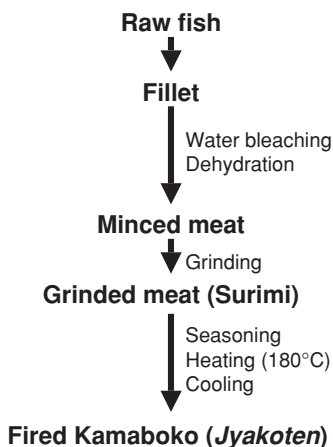
Fig. 32.1 Production of fried Kamaboko.

during the treatment. The generalized process of preparation of fried kamaboko – traditionally referred to as *Jyakoten* and specifi to the Ehime prefecture in Japan – is presented in Fig. 32.1. On measuring the quantities of EPA and DHA in the minced meat, ground meat or surimi (base material for preparation of the product), and *Jyakoten*, the amount of DHA slightly decreased during the grinding process, although EPA remained unchanged during this process. However, the frying step decreased the DHA content by 20%, but the quantity of EPA was not affected [34]. Contrary to the general notion that heating would affect the quantity of PUFA, the amounts of EPA and DHA rarely decrease during the heating process.

These results emphasized the fact that traditional products such as fried kamaboko could well have the EPA and DHA in the form and quantity desirable in the diet. The amounts of EPA and DHA seldom decreased during the drying process of traditional marine foods [35]. The results of changes in the amounts of EPA and DHA during the preparation of processed marine foods show that these traditional products are a good source of EPA and DHA, and seem to play a role in the prevention of obesity and life-style diseases.

32.3 Anti-obesity effect of histidine

32.3.1 Fish protein

It has been well documented that dietary proteins influenc lipid metabolism in humans as well as in animals [36–39]. Most of the research work dealing with the effect of dietary protein on lipid metabolism has evaluated the effect of plant proteins compared to that of casein as the animal protein source [37,40,41]. Furthermore, these works have established the fact that dietary soy protein has hypocholestermic and hypotriacylglycerolidemic effects. The hypocholestermic effect of soy proteins has been attributed to reduced intestinal absorption of cholesterol and enhancement of fecal cholesterol excretion [39]. Furthermore, its hypocholesterimic and hypotriacylglycerolidemic effects are believed to be due to modifica tion of lipid composition of microsomal membrane, alterations in fatty acid composition of membrane phospholipids, reduced activity of Δ -6 desaturase, and fatty acid synthase when compared to casein [42–44].

It is very important to note that besides casein and soy proteins, animal proteins in the form of beef, pork, mutton, poultry, and fis play an indispensible role in human nutrition. There are only a few reports regarding the effect of animal proteins [45], including marine proteins [46–48] on lipid metabolism as compared to soy protein or casein. Animal proteins including beef, pork, and turkey meat did not differ from casein in their effects on cholesterol metabolism [45]. Furthermore, it was also observed that most of these animal proteins did not alter concentration of cholesterol in plasma, lipoproteins, and liver, including the hepatic ratio of esterifie to free cholesterol as compared to casein. However, proteins from marine invertebrates *Anemonia viridis* and *Echinus esculentus* showed a hypocholesterimic effect when compared to casein and the same was attributed to alterations in intestinal enzyme activity [47,49].

Fish proteins improved blood pressure of spontaneously hypertensive rats [50,52]. Furthermore, hypocholesterolemic effects of fis proteins have been reported [48,51,52]. The main active components of fis proteins in its hypocholesterolemic effects were considered to be fis peptides formed by digestion [52]; however, the mechanism has not yet been elucidated.

32.3.2 Suppression of food intake by histidine

Recently, histidine is attracting increased interest due to its suppressive effect on food intake, which in turn results in the prevention of over-eating. Red fresh fish such as tuna, bonito, and sardines, contain large amounts of histidine in their proteins. Figure 32.2 shows the distribution of histidine in several foods. Generally, the amount of histidine/protein in meats is between 3 and 5%. It is interesting to note that histidine/protein in horse mackerel, Japanese pilchard, mackerel, and Pacifi saury are between 4 and 6%, whereas skipjack, yellowtail, yellowfi tuna, bluefi tuna lean meat, and bluefi tuna fatty meat are between 8 and 9%.

It is believed that dietary histidine is changed to histamine in the brain and acts to suppress food intake. Histamine is derived from histidine by means of histidine decarboxylase, which is present in the hypothalamus [53,54]. Histamine is reported to suppress the food intake by activating histaminergic neurons [55] that are located in the posterior hypothalamus [56]. Hypothalamic neuronal histamine, apart from its involvement in physiological homeostatic control of ingestive behaviour [57–60], is also reported to be involved in controlling mastication [60,61], and altering energy defici in the brain [58–64].

Sakata and colleagues, in the case of obese Zucker rats, reported one interesting and relevant observation *in vivo*. It is well accepted that obese Zucker rats provide an animal model of genetic obesity possessing behavioural [65] and metabolic [66–71] abnormalities such as over eating. The abnormalities in obese Zucker rats involve dysfunction of histaminergic neurons in the hypothalamus. It has been reported that the depletion of neuronal histamines in the hypothalamus mimicked the abnormalities of obese Zucker rats [67,72], and grafting of fetal hypothalamus of lean Zucker littermates into obese rats resulted in the attenuation of almost all the abnormalities [73].

In our own study involving the inhibitory effect of histidine-rich protein, on the food intake in students and adults living in a seaside area [4,74–77], the daily intakes of energy, protein, and histidine were surveyed. A negative correlation existed between energy intake and histidine/protein intake. Furthermore, the effect observed was more prominent in the female population than the male group. In another study, the effect of histidine on food intake was evaluated using a rat model [5]; we observed an alteration in food intake depending on the dietary histidine provided to the animals (Fig. 32.3). The food intake, each day, was significantl lower in rats provided with 5% dietary histidine compared to the group fed with 0% histidine. In addition, a significantl lower food intake was observed on days 4 to 6 and 8,
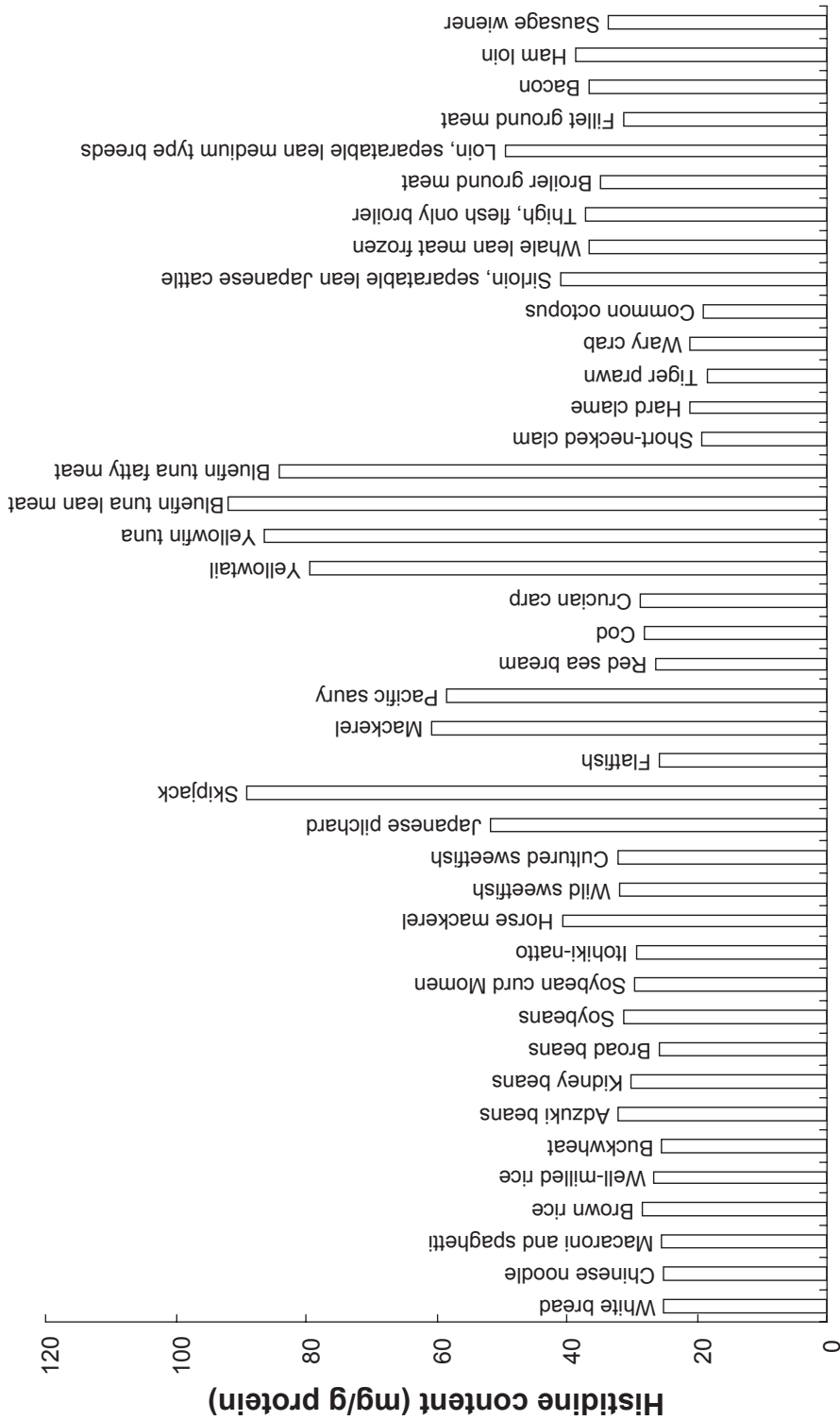
Fig. 32.2 Histidine content in several food. Adapted from Nakajima et al. [75], with permission of Japanese Society for Study Obesity.
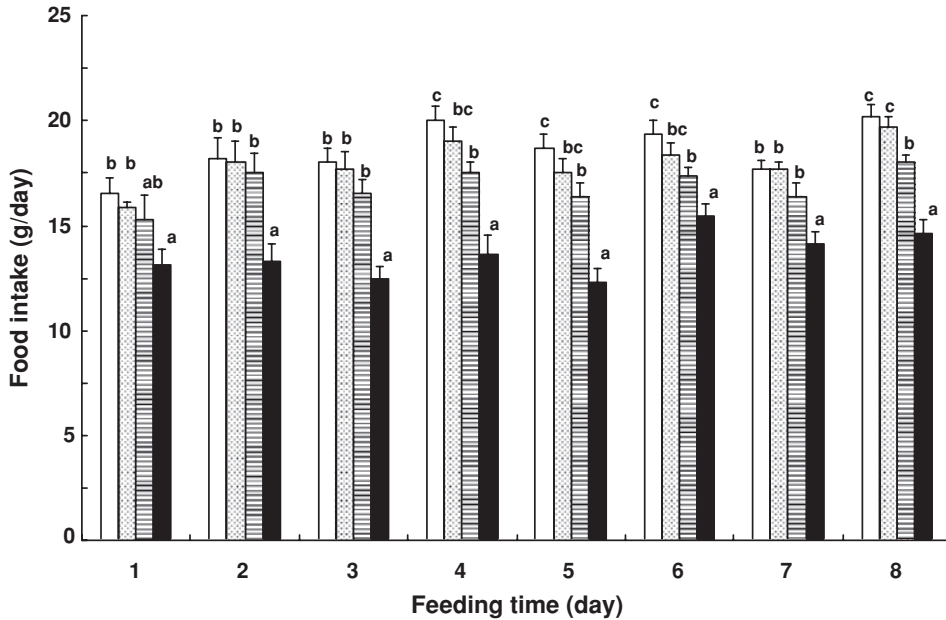
Fig. 32.3 Food intake in rats fed the 0, 1, 2.5, and 5% histidine diets for 8 days. White, dot, horizontal line, and black bars show 0, 1, 2.5, and 5% histidine, respectively. Each column value is the mean ± SD (*n* = 6). The values with different letters were significantly different from each other. *P* < 0.05. Adapted from Kasaoka *et al.* [5]). Copyright 2004, with permission from Elsevier.

in the group administered with 2.5% histidine. Food intake was slightly but not significantl lower in rats fed a 1% histidine diet than in rats fed a 0% histidine diet on days 4 to 6. There was a significan negative correlation between dietary histidine (g/8 days) and retroperitonial fat pads (g/100 g body weight) (*r* = −0.7064, *P* = 0.0007).

The significan lowering of food intake directly correlated (*r* = 0.5292, *P* = 0.0770) with increased UCP1 mRNA expression in animals administered with increased levels of dietary histidine (Fig. 32.4). UCP1 mRNA expression was also significantl higher (*P* = 0.0007) in rats fed the 5% histidine diet than those fed the 0% histidine diet. Furthermore, in another study, we observed the suppression of food intake and decreased accumulation of body fat in rats administered with dried bonito, in the form of a traditional product called *Katsuo-dashi*, as a source of histidine protein [78]. Based on our study in both animals and humans, it can be generally stated that oral administration of histidine-rich protein is useful to prevent over-eating and accumulation of body fat. These effects possibly result in the reported effects of histidine in prevention of obesity.

32.3.3 Underlying mechanism for effect of histidine

Histidine tastes slightly bitter to humans [79], and this bitter taste may help suppression of food intake. Alterations have been observed in food intake of rats fed standard, histidine-enriched or one of the four quinine diets [80]. The feeding pattern of the histidine-enriched diet group was quite different from that of the quinine diet groups. Food intake was suppressed immediately at 1 hour after feeding started in rats fed the 0.1 to 0.8% quinine diets. It appears that the bitter taste influence food intake very early during the

(a)

← UCP1

(b)

P = 0.0007

UCP1 mRNA (PSL)

Dietary histidine concentration (%)
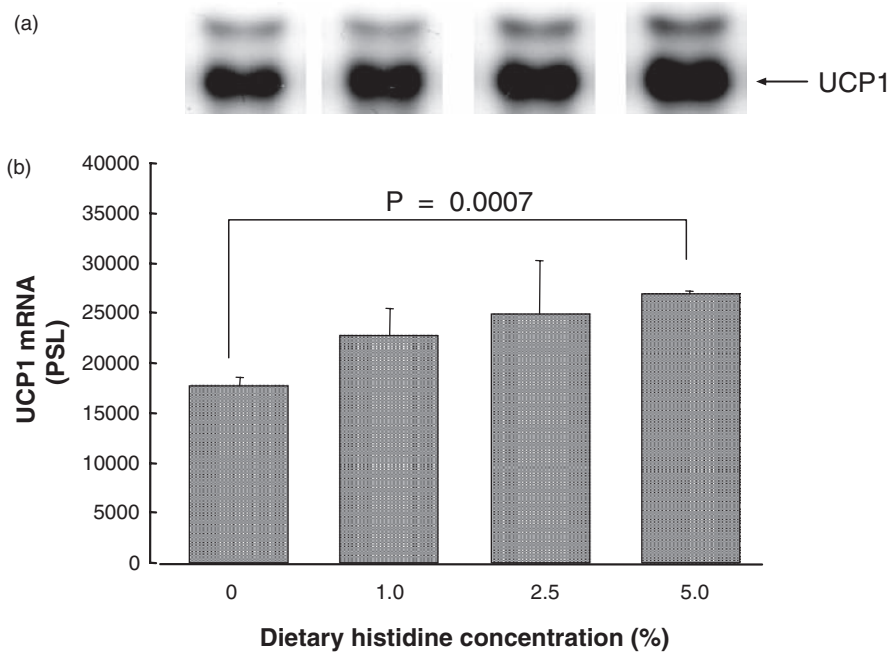
Fig. 32.4 Representative Northern blots (a) and phosphostimulated luminescence (PSL) (b) for messenger RNA of uncoupling protein 1 (UCP1) in brown adipose tissue of rats fed experimental diets. Each column value is the mean $\pm$ SD ($n = 3$). Adapted from Kasaoka *et al.* [5]). Copyright 2004, with permission from Elsevier.

feeding period. Food intake was somewhat high at 1 hour in rats fed the histidine-enriched diet compared with that of rats fed the standard diet. Thus, the bitterness of histidine might not play a role in food intake.

In order to clarify whether fluoromet ylhistidine (FMH), which inhibits conversion of histidine to histamine, affects food intake, Goto *et al.* [80] undertook further studies. Food intake significantl decreased in rats fed a histidine-enriched diet (12.6 g) in comparison with rats fed a standard diet (15.0 g) (Fig. 32.5). Contrary to expectation, food intake increased significantl in rats fed a histidine-enriched diet (14.1 g) and injected with FMH compared with those fed a histidine-enriched diet, but not injected with FMH (12.6 g). There was no significan difference in food intake between rats given the control diet and those given the histidine-enriched diet with FMH injection ($P = 0.1550$). Histamine derived from histidine activates the histaminergic neurons in the hypothalamus and thus decreases food intake [55]. By the time histidine is ingested orally, absorbed, and reaches the brain and is converted to histamine, several hours will have passed. This might explain why histidine slowly suppresses food intake after around 6 hours. It is known that brain histamine increases in rats after intraperitoneal administration of histidine [81]. In this study, food intake was restored to an amount almost equal to that of the standard diet by administration of FMH, an antagonistic inhibitor of histidine decarboxylase (HDC). These results suggest that synthesis of histamine is necessary for suppression of food intake by dietary histidine. Absorbed histidine might fl w smoothly into the brain, be converted to histamine by HDC, and distributed to the hypothalamus. Neuronal histamine might then stimulate the histaminergic neurons and suppress food intake. Thus, dietary histidine may suppress food intake through activation of histaminergic neurons regardless of its taste.
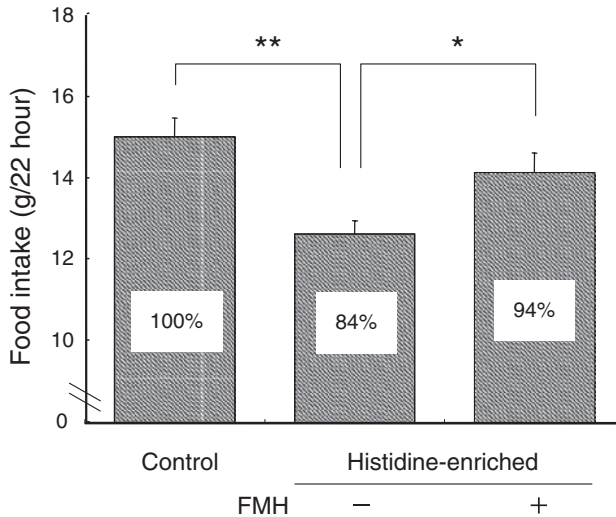
Fig. 32.5 Food intake of rats. Food intake (g/22 hours) in the rats ($n = 8$) fed the control diet, the histidine-enriched diet, and the histidine-enriched diet treated with FMH injection. *$P < 0.05$, **$P < 0.01$. Adapted from Goto *et al.* [80]. Copyright 2007, with permission from Elsevier.

32.4 Conclusions

Red fresh fis as well as processed food made from it contain large amounts of EPA and DHA in its lipids and histidine in its proteins. These marine foods appear to have a suppressive effect on obesity and life-style related diseases.

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33 Microencapsulation, nanoencapsulation, edible film, and coating applications in seafood processing

Subramaniam Sathivel and Don Kramer

33.1 Introduction

In general, every food goes through at least one mass transfer operation, whether it may be moisture transport, oxygen diffusion, losing aroma, or oil migration [1]. Films and coatings provide barriers to moisture, oxygen, fl vour, aroma, and oil to protect food quality from their surrounding environments. They can be formed from synthetic and biodegradable material, and those from biodegradable material are known as edible film and coatings and are expected to protect food quality similar to their synthetic counterparts. Their function may also be improved through the addition of antioxidants or antimicrobial agents. Edible film and coating technologies can be applied to seafood and fis oil to reduce lipid oxidation during storage, thereby extending their shelf-life. The permeability of edible film and coatings is important to their performance and this property is affected by the materials used in coating formulations. Materials used for forming film and coatings are mainly polysaccharides, lipids, proteins, or resins. Seafood and fis oils continue to undergo changes during storage, therefore, it is necessary to understand their biochemical reactions under different storage conditions before developing edible coatings for seafood and fis oils. This chapter focuses primarily on how to apply microencapsulation and edible coating technologies to fis oil and seafood to improve their shelf-life.

33.2 Application of microencapsulation technology in fish oil

Fish oils are the main source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are currently available in the marketplace. There is a large body of literature that describes the many positive benefit of dietary inclusion of long-chain omega-3 (ω-3 or n-3) fatty acids. The main sources of long-chain n-3 fatty acids are seafoods, including edible seaweed and algae. Fortificatio of foods with long-chain n-3 fatty acids is one approach used to increase their consumption. However, attempts to incorporate fis oil into food formulations has, in the past, met with limited success because of fis y fl vours in finishe products [2]. One of the technologies used nowadays for overcoming this problem

is microencapsulation. The microencapsulation process also makes it possible to transform these oils into powdered forms, wherein the small droplets of oil are surrounded by a shell coating of protein and/or carbohydrate. This results in small dry granules that have powder-like fl w characteristics. In the food industry, microencapsulation technology is used to entrap liquid droplets, solid particles, or gaseous compounds by using thin edible coating materials. A simple microencapsule consists of a core (nucleus) and coat (shell). The core may be one or several different types of food ingredients and the coating may be single- or double-layered. Shahidi and Han [3] have listed six reasons for using microencapsulation techniques in the food industry:

1) to reduce a core material reactivity with its surrounding environmental factors, such as temperature, moisture, and oxygen;
2) to reduce the diffusion rate of a core material to its surrounding environment through coating;
3) to control the diffusion of the core material to its surroundings;
4) to mask the core material's fl vour and taste;
5) to easily handle microencapsulated food ingredient;
6) to add only a small quantity of core materials to food products.

The selection of coating materials for fis oil microencapsulation should consider reduction of the release of fis oil to the microencapsule's surrounding environment. The wall materials should control the transport of oxygen, moisture, and air from the surrounding environment to the fis oil. Selection of coating materials for microencapsulation are typically based on the physicochemical properties, such as solubility, molecular weight, glass/melting transition, crystallinity, diffusivity, fil forming, and emulsificatio [4].

   Carbohydrates, such as starches, corn syrup solids, and maltodextrins, are considered to be good coating materials for developing microencapsulated fis oil powders. These coating materials exhibit low viscosities with high solid content and good solubility. The disadvantages of these materials are their lack of interfacial properties that are required for high microencapsulation efficien y. Therefore, they are materials generally associated with other encapsulating materials such as proteins or gums for producing microencapsules [4]. Gums are also used for microencapsulated core materials and they have fil forming and emulsion stabilization properties. Gum arabic, in particular, is widely used for producing microencapsulated powder due to its excellent emulsificatio properties. Proteins are re-garded as functional emulsifiers a property that makes them good coating components for preparing microencapsules. In addition, proteins possess high binding properties for fl vour compounds [4]. Whey protein and gelatin are most commonly used for microencapsulating food ingredients by spray drying.

   Spray drying, spray cooling, fluidize bed drying, extrusion, and centrifugal extrusion are among of the techniques used to produce microencapsules. However, the spray drying process is the most cost-effective way to produce microcapsules in a relatively simple and continuous processing operation [5]. Shahidi and Han [3] have reported that application of the spray drying process for the production of microencapsules involves four stages:

1) preparation of the dispersion or emulsion;
2) homogenization of the emulsion;
3) atomization of the feed emulsion; and
4) drying the atomized particles.

It is important to form a stable emulsion of fis oils in the coating solution. The dispersed coating material must be homogenized. Physicochemical factors such as pH, ionic strength, and the ratio of coating materials, affect the formation and stability of emulsions containing fis oil. The temperature, pressure, and shearing rate applied to an emulsion system also influenc phase separation and time stability of an emulsion containing fis oil. It is important to keep emulsions stable over a certain period of time before spraying any emulsion into a spray drying chamber to observe the stability of the emulsion system [6]. Emulsions containing fis oils are atomized as wet particles in heated air streams that are supplied to the drying chambers of the spray dryer. During such spray drying operations, water evaporates from the atomized particles that lead to the formation of microencapsules [7].

The oxidative stability of fis oil is improved by using microencapsulation processes. In addition, the microencapsulation of fis oil can impart many benefit such as providing an oxygen barrier resulting in an extended shelf-life, a taste profil barrier eliminating fis oil taste and odour, nutritional availability, and a protective barrier from shear and temperature changes when incorporated into food products. Microencapsulated n-3 fatty acids can be used in a wide assortment of foods. For example, Novomega, an n-3 fatty acids encapsulated product is marketed for use in the bakery products [8]. The encapsulation system of the Novomega is specially formulated for long-chain n-3 fatty acids, and results in a product that eliminates strong fis oil tastes and odours. Two other fis oil encapsulated powders, Marinol^{TM} Omega-3 HS and Marinol DHA HS, are marketed in the US. Another n-3 microencapsulated fis oil powder, MEG-3 has been introduced in the Canadian and US markets. These powders have been included into bakery, milk, and beverage markets. Yin *et al.* [9] reported that microencapsulated red salmon oil powder contains 42% red salmon oil, 13.0 mg DHA/g powder, 13.9 mg EPA/g powder, and 41.4 mg n-3 fatty acids/g powder. Particle diameter of the salmon oil powder ranged from 5.5 to 88 μm. The particle size of microencapsulated powder usually ranges from 1 to 2,000 μm [10]. Particle size of the microencapsulated fis oil depends mainly on the physical properties of emulsions, such as viscosity, density, and concentration. The viscosity of the fis oil emulsion affects particle size distribution, because the viscosity of the emulsion interferes with spray drying atomization. Rosenberg *et al.* [11] have reported that high viscosities of emulsions produce large droplets and affect drying rates. To obtain high-quality microencapsulated fis oil powders, one must identify the best coating material and optimum spray drying conditions. Feed temperature, feed fl w rate, air inlet temperature, and air outlet temperature are always optimized in spray drying operations, in order to obtain the highest quality microencapsulated products possible.

33.3   Nanoencapsulated fish oil

Nanotechnology is a new frontier of this century, but its application to the food sector is relatively recent compared with application uses in drug delivery and pharmaceuticals [12]. Nanotechnology can play a major role in a food system to deliver effectively more nutrients and bioactive compounds to the human body [13]. Fletcher [14] has reported that the world sales of nanotechnology products are expected to reach US$20.4 billion by 2010. Chau *et al.* [15] have reported that nanotechnology research for food and food related products is just starting to develop.

In recent years, most food industry tends to develop food products containing bioactive compounds to reduce the risk of food-related illnesses such as cardiovascular disease (CVD), hypertension, and cancer [16]. Inclusion of fis oil or n-3 fatty acids is essential

for production of certain functional foods. According to Weiss *et al.* [17], nanoencapsules have to be engineered to incorporate functional component such as n-3 fatty acids in a food system. Nanoencapsulation system provides physical stability and protects n-3 fatty acids against chemical degradation. It controls the release of encapsulated n-3 fatty acids during mastication and digestion to maximize absorption. Several major contrasts can be identifie between nano- and microencapsules [17]. Microcapsules are often thermodynamically unstable and tend to break down over time. They are relatively larger than nanoencapsules, which particularly slows down absorption and also release encapsulated compounds, making them far superior to microencapsules [17]. Nanoencapsulated fis oil may offer the possibility of delivery and transport of n-3 fatty acids though mucosal surfaces after oral administration. Nanoencapsulated fis oil powder is expected to be more biologically active than microencapsulated fis oil. By reducing particle size of the fis oil powder, the delivery properties, solubility, prolonged residence time in the gastrointestinal tract, and efficien absorption through cells can be enhanced. Nanoencapsulated fis oil can be produced in a similar manner to microencapsulated fis oil by coating oil with bioploymers. A simple nanoencapsulated fis oil can be produced by the following steps, as described by Preetz [18].

1) A micron-sized emulsion is obtained by dispersing the fis oil in the coating solution.
2) The size of the emulsion is reduced by high-pressure homogenization.
3) Dispersion is centrifuged and separated from the aggregated emulsion.

Natural polymers such as albumin, gelatin, alginate, collagen, chitosan, and milk protein can be used as a coating material to prepare nanoencapsulated fis oil. Chen *et al.* [19] have reported that nanocapsules can be relatively easily prepared using protein based coating materials.

Determination of nanoencapsule size and surface charge is important. The size of the nanoencapsules can be determined by transmission electron microscopy, photon correlation spectroscopy, and static light scattering. The surface charge of the nanodispersions can be characterized by the measurement of the zeta potential. Nuclear magnetic resonance (NMR) spectra can be recorded to investigate the physicochemical status of the nanocapsule components.

Chen *et al.* [20] have reported that nanotechnology will have a great potential for improving the effectiveness and efficien y of delivery nutraceuticals and functional foods to improve human health. Little information is available on nanoencapsulated fis oils, which may facilitate controlled release and improved bioavailability of fis oils containing n-3 fatty acids. Understanding the mechanism of target delivery will enable fis oil manufacturers to design smart nanoencapsulated products for delivering the optimal level of n-3 to each individual.

33.4   Edible film and coating applications in seafood

In general, edible film and coatings are biodegradable and are intended to function as barriers to moisture, oxygen, fl vour, aroma, and oil; thus, food quality and shelf-life can be improved. There has been a resurgence of interest in the development of edible film and coating materials from seafood by-products. Avena-Bustillos *et al.* [21] recently evaluated the water permeability properties of film made from fis skin gelatin, including pollock

gelatin. Paschoalick *et al*. [22] reported that the myofibrilla and sarcoplasmatic proteins obtained from fis could be used as ingredients to form edible films

Edible packaging film carry functional ingredients such as antioxidants or antimicrobials. The film also improve appearance, structure, and handling properties. Edible film can be used as wraps for foods to protect them from mechanical damage during transportation or handling and to delay lipid oxidation of unsaturated fatty acids in food due to the film s low oxygen permeability and the antioxidative properties.

Oxygen, carbon dioxide, nitrogen, and water permeabilities of edible fil are among key factors that limit the shelf-life of packaged or coated products. In general, oxygen, carbon dioxide, nitrogen, and water vapour transport, in steady-state conditions through a film can be described by Fick's law.

Edible coatings can also be applied to seafood. Fish is an extremely perishable food compared to other fresh commodities, therefore freezing is a general preservation method used to control or decrease biochemical changes in seafood that occurs during storage. However, frozen storage does not completely inhibit chemical reactions (e.g. lipid oxidation) that lead to quality deterioration of fish Preservatives such as phosphates are often used to prolong the shelf-life, increase water binding capacity, reduce oxidation, and affect other properties in seafood and meat products. Phosphates are used to enhance the water holding capacity of seafood, and to improve their cooking yield [23]. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been commonly used as antioxidants by the food industry to extend product shelf-life and improve quality of products. There is a great consumer interest in natural ingredients that enhance food quality and shelf-life. Biodegradable natural ingredients, including polysaccharides and proteins, can be used as fis and meat coatings to control quality deterioration during frozen storage.

Biodegradable ingredients, such as polysaccharides and proteins, can be used to coat fis fillet to suppress quality changes during frozen storage. Some biodegradable edible coatings applied on frozen foods act as a barrier to control moisture transfer and oxygen uptake [24]. Several coating materials have been tested in an attempt to maintain quality and prolong shelf-life of meat and fis products. Stuchell and Krochta [25] have reported coated king salmon with whey protein isolates in order to delay lipid oxidation in the product during frozen storage. Sathivel [26] has evaluated the effects of 1 and 2% chitosan solutions, egg albumin, soy protein concentrate, pink salmon protein powder, and arrowtooth flounde protein powder as edible coatings on the quality of skinless pink salmon fillet during 3 months of frozen storage. Coating with 1 and 2% chitosan is effective in reducing about 50% relative moisture loss, compared to the control non-coated fillet (Fig. 33.1).

Lipid oxidation is a major problem during storage of seafood that can be measured using the thiobarbituric acid reactive substances (TBARS) value. Coating with 1 and 2% chitosan and soy protein concentrate has been reported to be effective in delaying lipid oxidation [26] (Fig. 33.2). The antioxidant properties of chitosan in foods have been reported [27]. Jeon *et al*. [28] have also reported that chitosan coatings reduce lipid oxidation in herring and Atlantic cod. Weist and Karel [29] have reported that the primary amino groups of chitosan will form a stable fluorospher with volatile aldehydes, which are derived from the breakdown of lipids during the oxidation. Chitosan coatings and film have been reported to serve as good barriers to oxygen permeation [30]. Sathivel *et al*. [31] have reported that the oxygen permeability coefficien of the chitosan fil is $5.34 \times 10^{-2} \pm 0.002$ cm^3/m day atm, which is higher than the reported oxygen permeability coefficien value (7.2×10^{-5} cm^3 O$_2$/m day atm) [32]. Caner *et al*. [33] have reported that oxygen permeability of chitosan fil ranges from 0.08 to 31.07×10^{-3} cm^3 O$_2$/m day atm, which is affected by the type of acids and
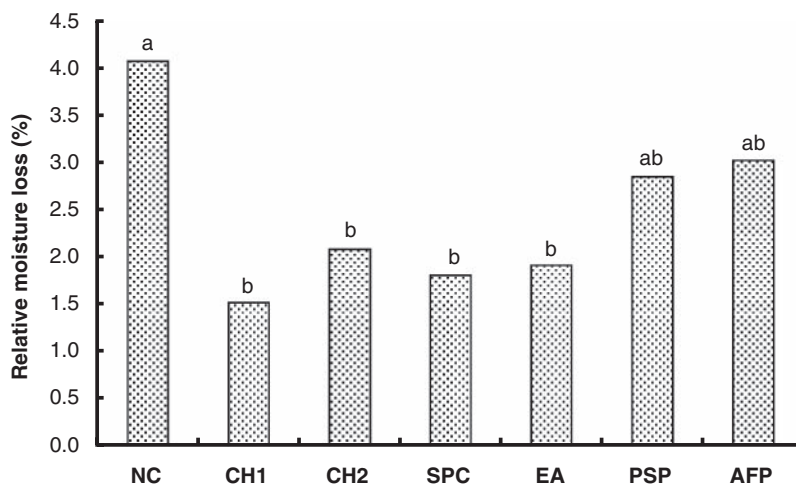
Fig. 33.1   Relative moisture loss of pink salmon fillets during a 3 months frozen storage. [a-b]Means with the same letters are not significantly different ($P > 0.05$). Abbreviations: NC, control; CH1, salmon fillets coated with 1% chitosan; CH2, salmon fillets coated with 2% chitosan; SPC, salmon fillets coated with soy protein concentrate; EA, salmon fillets coated with egg albumin; PSP, salmon fillets coated with pink salmon protein powder; AFP, salmon fillets coated with arrowtooth flounder protein powder. Adapted from Sathivel [26], with permission of John Wiley & Sons, Inc.

plasticizer concentrations used for preparing chitosan films Therefore, 1 and 2% chitosan coating applied on the surface of pink salmon fillet may have acted as a barrier between the fille and its surrounding environment, thus slowing down the diffusion of oxygen from the surrounding environment to the surface of the fillet Soy protein concentrates coating salmon fillet have also been found to be very effective in controlling lipid oxidation by serving as a barrier to O_2 permeability [34].
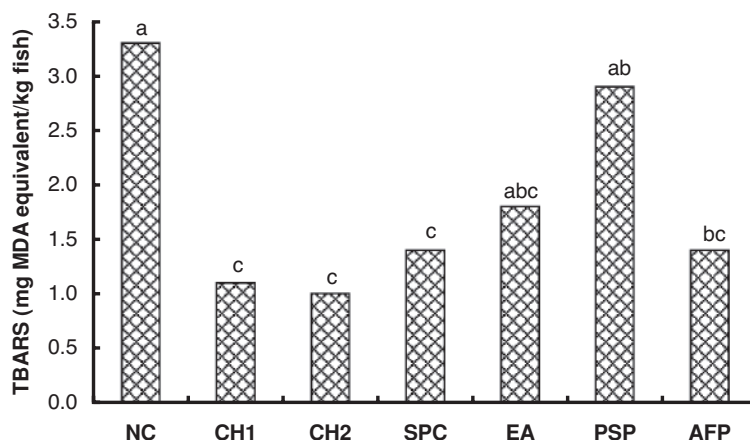
Fig. 33.2   Thiobarbituric acid reactive substances (TBARS) of pink salmon fillets during a 3 months frozen storage. [a-c] Means with the same letters are not significantly different ($P > 0.05$). Adapted from Sathivel [26], with permission of John Wiley & Sons, Inc.

The arrowtooth flounde fis protein coated salmon fillet showed reduced lipid oxidation activity similar to that of fillet coated with 1% chitosan, 2% chitosan, and soy protein concentrate (Fig. 33.2). This is likely due to the antioxidant activity of peptide fractions in the arrowtooth flounde proteins. Fish protein hydrolysate has been reported to have antioxidant properties [35]. Shahidi and Amarowicz [36] have reported antioxidant activity of protein hydrolysates from two aquatic species, namely capelin and harp seal. Kim *et al.* [37] have reported that pollock skin protein hydrolysates have antioxidant and other functional properties. Sathivel *et al*. [38] have reported that coating solution prepared from pollock skin hydrolysate acts as an antioxidation agent and delayed lipid oxidation of pink salmon fillet during four months of frozen storage. Peptides generated during hydrolysis coupled with free amino acids could be responsible for the antioxidant activity [39]. The effectiveness of food surface coatings can be influence by a number of factors, such as coating permeability, thickness, and surface coverage by the coating materials.

33.5 Conclusions

Microencapsulation and edible coating technologies can be used to improve the shelf-life of fis and seafood. Microencapsulated fis oil can fulfi the physical and nutritional re-quirements of the ingredient for enrichment of a variety of foods, such as milk and bakery products, salad dressings, juices, and nutraceutical markets. The microencapsulated powders may appeal to broader groups of consumers when applied to snacks, breads, cookies, pizza toppings, and other products such as chicken nuggets and meat sausages. Nanoencapsulated fis oil may deliver and transport n-3 fatty acids though mucosal surfaces after oral adminis-tration. The natural antioxidant of fis skin protein and chitosan make them ideal as coating materials to suppress lipid oxidation in fis fillet during frozen storage. Edible coatings prepared from fis skin and chitosan have potential applications for enhancing the storage stability and quality of frozen fillets

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34 Fish oil extraction, purification, and its properties

Subramaniam Sathivel

34.1 Introduction

With the increasing demand for fis oil as a healthy and functional food, the quality of fis oil is becoming extremely important to the seafood industry. Fish oil is an abundant source of polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). For the last two decades, interest in dietary effects of marine omega-3 fatty acids (n-3 or ω-3) has increased, because they play a major role in human health. Natural fis oils have been claimed to help maintain heart and vascular health in humans [1]. Producing and purifying fis oil from whole fis and/or fis processing by-products for the growing fis oil market can benefi from the seafood industry. Crude oil can be extracted from whole fis and/or fis processing by-products, including viscera, heads, skins, frame, and discarded fish Fish by-products are obtained from the edible fisherie industry such as cuttings from filletin operations, fis cannery waste, roe fishin waste, and surimi processing. Menhaden oil is extracted from whole menhaden while salmon and pollock oils are extracted from their processing by-products. This chapter covers fis oil extraction, purification and their thermal and rheological properties.

34.2 Extraction

The conventional method of extracting oil from fis is rendering, a process in which high heat is used to extract fat or oil mainly from animal tissues. Almost all animal fats are recovered by rendering, whereas vegetable oils are obtained by crushing/pressing or solvent extraction or both. In general, rendering can be conducted under wet or dry conditions. Wet rendering is carried out with large amounts of water. The fat cell walls are broken down by steam under pressure until they are partially liquefie and the released fat float to the surface of the water. Separated fat is removed by skimming or by centrifugal methods. The wet rendering is a universal process and used in the majority of the fis oil industry. Fish oil industries all over the world (both on land and in ships) use this method. They may have slight differences in equipment type, but the major steps of cooking, pressing, and separating are always present [2].
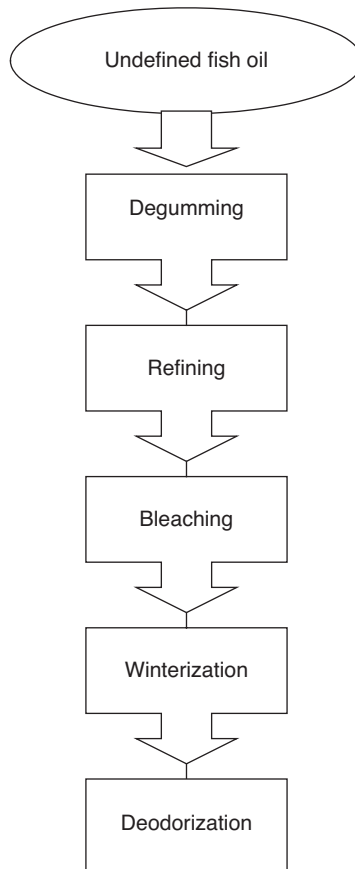
Fig. 34.1 Production of unrefined fish oil from a typical fish meal plant.

In general, fish meal plants produce fish oil from fish by-products and discarded fish where the by-products are ground and cooked for 20 minutes at 95°C. The cooked fish by-products are then mechanically dewatered in a screw press, which subsequently produces press cake and press liquor. The press liquor is clarified using a decanter to remove suspended solids and then the clarified liquor is centrifuged to separate the oil and the aqueous fraction.

Extracted fish oil contains free fatty acids (FFA), oxidation products, minerals, pigments, moisture, phospholipids, and insoluble impurities that reduce oil quality. Removal of impurities from crude fish oil is very important for producing purified oil with desirable and acceptable shelf-life. Conventional fish oil refining steps are degumming, neutralizing (alkali refining) bleaching, winterizing, and deodorizing (Fig. 34.1). Degumming removes phospholipids; neutralization removes FFA which are precipitated as soap; bleaching uses clay to adsorb pigments and breakdown of the primary oxidation products; and finally, deodorization removes oxidized components along with other volatiles. Each step is given in detail below.

34.2.1 Degumming

Degumming is a treatment designed to remove the impurities such as phospholipids, FFA, and trace metals, with the least possible damage to the natural oil. The impurities are complex

molecules and are generally classifie as "gum". The presence of gum in the oil determines the fina oil colour, fl vour, foaming, and smoking stability of oil and it must be removed. Occasionally, the gum settles out in a storage tank and can cause high refinin losses. The gum can be removed from the oil with water or acids such as phosphoric acid or citric acid, etc. [3–5].

The gum in the oil is broadly divided into hydratable and non-hydratable types. The hydratable type is treated with water or steam and converted to a hydrated gum, which is insoluble in the oil and separated by centrifugation [3]. For water hydration processes, the crude oil is heated to 70°C and 1 to 3% of water by weight is mixed into the oil. The mixture is agitated slowly for about 30 minutes and then centrifuged to remove the gum. The non-hydrated gum is usually removed by treating the oil under vacuum with acids such as phosphoric or citric acid. In acid degumming, the oil is heated to 70 to 85°C and about 0.1% (weight basis, wb) phosphoric acid and/or 0.3% (wb) citric acid is added and intensively mixed for about 30 seconds. After an additional 15 minutes agitation by a stirrer, 1% (wb) demineralized water is added to the acid-in-water emulsion and agitated for another 15 minutes and the oil is then removed by centrifugation [6].

Degumming is not ordinarily carried out in fis oils processing, because they have a very low amount of phosphatides. In some refineries however, an acid pre-treatment, designed to hydrate gums and remove phosphorous and other trace metals, is applied to oil as it enters the alkali refinin plant. The pre-treatment of fis oils with phosphoric acid prior to caustic refinin is a standard practice in Europe [7]. Degumming has been shown to effectively reduce lead, copper, arsenic, and zinc in menhanden oil [8,9].

34.2.2   Neutralization (alkali refining)

Neutralization is a purificatio process designed to remove non-acylglycerol impurities such as FFA in the oil. In general, the FFA can be removed from oils in two ways, chemical (neutralization) and physical methods. In the case of neutralization, an alkali solution is added to crude oil. The alkali combines with FFA present in the oil to form soaps [5]. The alkali most commonly employed for neutralization oils is caustic soda. However, it saponifie some of the neutral triacylglycerols (TAG), causing a higher purifying loss [5].

Another method to remove FFA and volatile components is the physical method. Since FFA are more volatile than glycerols, it is also possible to remove them from the oil by high-temperature steam distillation [10]. This process is called "physical refining" However, fis oils are not normally physically refine because they are too unstable. The highly unsaturated TAG would polymerize during the distillation and produce a rapid fl vour reversion after refinin [2].

Fernandez [11] explained a cation strong-acid microporous resins process for the refinin of fis oil for human consumption. Conventional refinin processes require high temperature treatment that damage n-3 fatty acids in fis oil. On the other hand, resins that do not require high temperature, are of consistent quality and can be regenerated.

34.2.3   Bleaching

Bleaching is designed to improve colour, off-fl vour, and oxidation products [12]. Many compounds in crude oil responsible for the colour are broken down at high temperatures and the volatile products are removed under deodorization conditions. Bleaching involves the adsorption of coloured compounds in the oil by activated clay [2]. The bleaching step is also

important to remove soap, trace and heavy metals, and sulphur compounds. During bleaching, peroxides are broken down to aldehydes and ketones and these secondary oxidation products are adsorbed onto the activated earth surface such that the filtere oil after bleaching should have a low peroxide value (PV) compared with the oil before bleaching.

Adsorption is commonly used for bleaching and it is done by transferring the flui phase to the surface of a solid adsorbent. Usually the small particles of adsorbent are held in a fi ed bed, and the flui is passed continuously through the bed until the solid is nearly saturated and the more desired separation can no longer be achieved. The fl w is then switched to a second bed until the saturated adsorbent can be replaced or regenerated [13].

The two types of commercial bleaching clays used in processing of edible oils are characterized as "Natural Bleaching Earth" and "Activated Bleaching Earth" [14]. Natural bleaching earth, also known as Fuller's earth, is basically a hydrated aluminium silicate. In recent years, there has been considerable interest in acid activated clays for bleaching of oil and fats. The raw materials used for the manufacture of this type of bleaching clay consists of mostly bentonite or montmorillonite, which have little or no decolorizing power in the raw state. In general, the clays are treated with sulphuric or hydrochloric acid. The acid treatment extends the surface of the clay and causes important changes in its chemical or physicochemical nature. Acid-activated clays retain more oil per unit weight of clay than do natural earths.

34.2.4 Deodorization

Deodorization is one of the major processing steps in the refinin of edible oils. Due to current harvesting and processing practices, high concentration of PUFA, and other contaminants, crude fis oils are easily subjected to deterioration. This severe deterioration changes the fl vour quality of fis oils. Off-odours and fl vours in fis oil arise from metabolite contaminants, from fis oil protein spoilage, or from oxidation of the fis oil itself [15,16]. Hsieh *et al.* [17] studied the volatile components of crude winterized menhaden oil by dynamic headspace/gas chromatography (DH/GC) analysis and found that many odour components are derived from lipid oxidation, including short-chain saturated and unsaturated aldehydes, ketones, and carboxylic acids.

Undesirable ingredients produced by previous refining bleaching, hydrogenation, or even storage conditions may affect the fl vour quality of fis oil. Therefore, undesirable odours and volatile components should be removed during refinin and deodorization to obtain food grade oil with good cooking quality. Deodorization has been considered as a unit process that finall establishes the oil fl vour and odour characteristics that are most readily recognized by the consumer [18–20]. Steam deodorization is possible because of the great differences in volatility between the TAG and the substances that give oils and fat their natural fl vours and odours. It is essentially a process of steam distillation where the volatile compounds are stripped from the non-volatile oil [21].

34.2.5 Fractionation or winterization

Fractionation or winterization operations in the processing of edible oils are basically the separation of oils into two or more fractions with different melting points [22]. Melted oils are directly cooled and allowed to form crystals, and the crystalline mass is separated from the remaining liquid fraction [23]. The objective of winterization processes is to remove

trisaturated and disaturated glycerides, waxes, and other non-triglyceride constituents. It is a slow process, and the entire winterization process from start to finis takes up to six days. Solvent can also be added to oils for winterization. In this process, the oil viscosity is reduced by means of a solvent such as hexane. Fish oils are cooled in the presence of solvent yielding high-melting crystals that are separated by filtratio [24].

34.3   Fish oil properties

Knowledge of thermal, rheological, and oxidation properties of the fis oil is essential for the design of a proper refinin process, the analysis of production costs, and the fina quality evaluation. The differential scanning calorimetry (DSC) is used to determine the quality of catfis and menhaden oils at different purificatio steps [25].

   DSC offers a simple method to investigate the characteristics of melting of fis oil. The influenc of oil composition, water content, production materials, aging, and heat treatment on the oil quality can be demonstrated using DSC [26]. It has been used to investigate the thermal conductivity, specifi heat [27], melting, crystallization [28,29], oil content [30], wax coating [29], and phase transition [31] of foods. Knowledge of rheological properties of the fis oil helps to solve problems related to the transfer or movement of bulk quantities of the oil. During refining impurities, such as FFA, proteins, moisture, pigments, and volatile compounds, are sequentially removed from the crude unrefine fis oil [32]. Removing impurities may alter the rheological properties of the oil [33].

   Marine oils, which contain higher quantities of PUFA, are susceptible to oxidation [34]. Lipid oxidation of the oil mostly depends on the storage temperature and time [26,35]. The temperature dependent viscosity and oxidation of fis oil could be extrapolated by using the Arrhenius equation, which expresses the relationship between the rate constant and the activation energy of a reaction [36].

34.3.1   Thermal properties of fish oil

Fish oil is subjected to temperature changes during the extraction and purificatio processes. Due to temperature changes, the overall physical and chemical properties of oil may drastically alter the fina oil quality [37]. Temperature changes are associated with enthalpy, which explains whether oils change from one physical state to another by absorbing (endothermic) or releasing (exothermic) heat [38]. Thermal properties related to temperature changes, such as melting point, enthalpy, and specifi heat, of fis oil can be measured using a DSC. Melting of fis oils depends on the composition of the unsaturated and saturated TAG presents. Sathivel *et al*. [39] reported that the melting point of the unrefine Pollock oil ranged from –69.5 to 14.2°C, while the melting points ranged from 69.6 to –0.36°C and –64.7 to 20.8°C for red and pink salmon oils, respectively [40]. Investigators found that the negative melting points of fis oils were attributed to TAG, which contained unsaturated fatty acids [25,26]. Oil samples with a higher degree of unsaturated fatty acids melt at negative temperatures, whereas those with a higher degree of saturated fatty acids melt at higher temperatures. The sharpness of melting points of fis oil depends on impurities present in the oil. Refine fis oil has a sharper and narrower peak in the DSC thermograms, whereas the unrefine fis oil shows broad peaks [41]. The melting points of fis oil are sharper after each purificatio step that removes impurities from the oil [25].

34.3.2 Rheological properties of fish oil

Information on the rheological properties is essential in controlling flui transfer, and purifi cation of fis oils. During purification impurities such as FFA, proteins, moisture, pigments, minerals, oxidation products, and volatile compounds, are sequentially removed from crude fis oil [32]. Removing impurities may alter the rheological properties of the fis oils [33]. Precise information on rheological properties provides better control over flui velocity, pump pressure, and energy consumption during the production of oil. The power law model (Eqn. 34.1) can be used to analyze the rheological properties of fis oils:

$$\sigma = K\gamma^n \tag{34.1}$$

where σ = shear stress (Pa); γ = shear rate (s^{-1}); K = consistency index (Pa.s^n); and n = fl w behaviour index.

Huang and Sathivel [42] investigated the rheological properties of unrefine salmon oil between 0 and 35°C (with 5°C intervals). The fl w behaviour index (n) of the unrefine salmon oil sample ranged from 0.8 to 0.88, which indicates its slight non-Newtonian be-haviour (Table 34.1). The consistency index (K) value for the unrefine salmon oil is higher at lower temperatures. A relationship can be obtained between the apparent viscosity of fis oils and their purificatio or handling temperature using the Arrhenius equation (Eqn. 34.2):

$$k = Ae^{(-E_a/RT)} \tag{34.2}$$

where k is the reaction rate constant; A is the frequency factor; E_a is the activation energy (J/mol); R is the gas constant (8.314 J/mol K); and T is the temperature (K).

This equation can be used to calculate the average magnitude of activation energy of the unrefine salmon oil from 1/T and the natural logarithm of the apparent viscosity. The E_a indicates the energy barrier that must be overcome before the elementary fl w process can occur [43]. The magnitudes of Ea (21.80 kJ/mol) and A (5.7×10^6) for the unrefine salmon oil are given in Table 34.1. The degree of fit as shown by the r^2 value of 0.98, indicated that changes in apparent viscosity with temperature could be well described by the Arrhenius

Table 34.1 Flow behaviour index, consistency index, apparent viscosity, and Arrhenius parameters of unrefined salmon oil. Adapted from Huang and Sathivel [42]. Copyright 2008, with permission of Elsevier

Temperature (°C)	n	K (Pa.s^n)	Apparent viscosity (Pa.s)
5	0.88 ± 0.02^a	0.148 ± 0.016^a	0.071 ± 0.001^a
10	0.85 ± 0.05^{ab}	0.132 ± 0.022^{ab}	0.063 ± 0.001^b
15	0.86 ± 0.01^{ab}	0.110 ± 0.010^{bc}	0.048 ± 0.001^c
20	0.84 ± 0.00^{ab}	0.108 ± 0.004^{bc}	0.042 ± 0.001^d
25	0.82 ± 0.01^{ab}	0.107 ± 0.006^{bc}	0.036 ± 0.001^e
30	0.80 ± 0.01^b	0.105 ± 0.010^{bc}	0.032 ± 0.000^f
35	0.80 ± 0.01^b	0.097 ± 0.006^c	0.030 ± 0.000^g
E_a(J/mol)			21803.04 ± 373.01
μ_∞			5.70E-06 ± 7.65E-07

Values are means ± SD of three determinations.
Abbreviations: **n** = flow behaviour index, **K** = consistency index, **Ea** = activation energy, μ_∞ = the frequency factor.
$^{a-g}$Means with the same superscript letter in each row are not significantly different ($P > 0.05$).
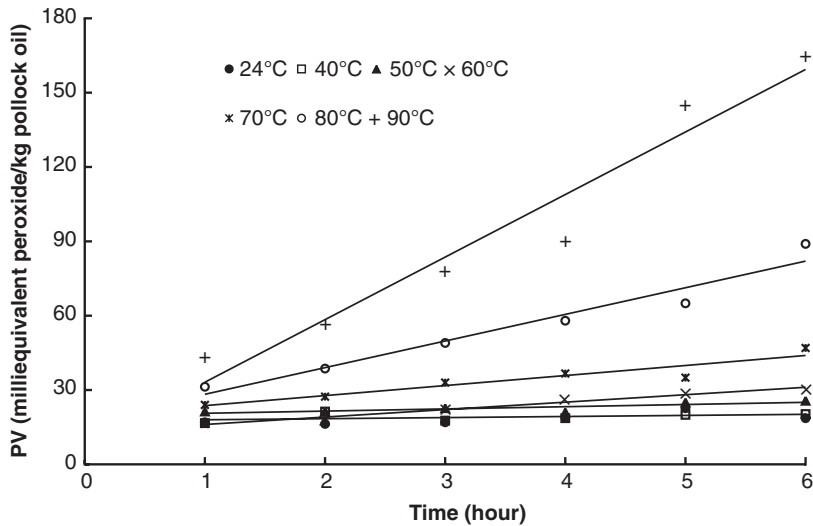
Fig. 34.2 Peroxide values of the unrefined pollock oil at different temperatures. Adapted from Sathivel *et al*. [39]. Copyright 2008, with permission of Elsevier.

equation. This study shows that the magnitude of apparent viscosity of unrefine fis oil is greatly influence by temperature.

Lipid oxidation is a major problem during extraction, purification and storage of fis oil. Sathivel *et al*. [39] reported that changes in lipid oxidation of pollock oil depended on the storage period and storage temperature. PV measures the formation of hydroperoxides, which are primary products of oxidation. Lipid oxidation, as indicated by the PV values, increases with increased time and temperature (Fig. 34.2). The unrefine Pollock oil stored at 24°C for 6 hours exhibited minimal lipid oxidation, whereas those oils at 70 to 90°C showed higher lipid oxidation after 6 hours. In addition, Sathivel *et al*. [39] reported an increase in the formation of primary oxidation products in the unrefine pollock oil with increased storage time at 4, 24, and 40°C (Fig. 34.3). The rate of hydroperoxide formation in the oil stored at lower temperature (4°C) was lower than that of the oil stored at room temperature (24°C). Frankel [44] reported that the formation rate of hydroperoxides at lower temperatures was attributed to the lipid and antioxidant concentration in the oil, but at elevated temperatures the rate was mainly related to oxygen concentration. The oil stored at 40°C (Fig. 34.3) has a slower rate of hydroperoxide formation than that of the oil stored at 24°C; this might be attributed to lower oxygen solubility at an elevated temperature. Furthermore, the rate of decomposition of primary hydroperoxides is higher at an elevated temperature; therefore, they do not accumulate in the oil stored at 40°C compared to those at 24°C. Aidos *et al*. [35] reported a similar pattern of formation and decomposition of hydroperoxidise for herring oil stored at elevated temperatures.

34.4 Conclusions

Information on thermal and rheological properties and lipid oxidation of unrefine fis oil is useful for designing the process and optimizing unit operations for purificatio process,
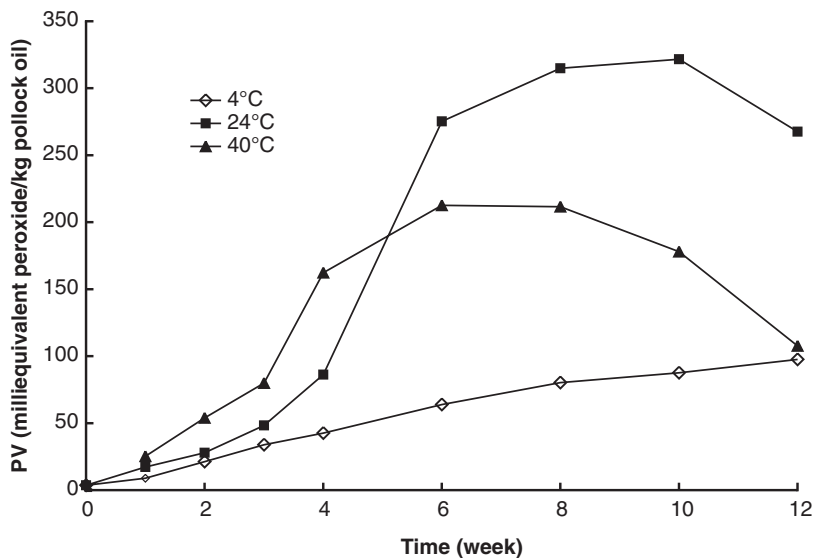
Fig. 34.3 Peroxide values of the unrefined pollock oil during 12 weeks of storage. Adapted from Sathivel *et al.* [39]. Copyright 2008, with permission of Elsevier.

analysis of production cost, and fina quality evaluation. As each purificatio step of fis oil involves different temperature conditions, it is important to be able to predict apparent viscosity of the fis oil at each purificatio step. The changes in the magnitude of apparent viscosity of the unrefine fis oil with temperature could be well described by the Arrhenius equation. The rate of lipid oxidation of the fis oil can be influence by both storage time and temperature.

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35 Nutraceutical quality of shellfish

Bonnie Sun Pan

35.1 Introduction

The Greek physician Hippocrates, the father of medicine in the 4th century, advised "Let food be your medicine and medicine be your food." In traditional Chinese concept, medicine and food are isogenic. Epidemiological studies provide convincing evidence that dietary factors can modify carcinogenesis during initiation, promotion, and progression of human cancer [1,2]. Shellfis is an example that has been considered a remedy for jaundice, hepatitis, and liver diseases. However, only a few scientifi research studies have been published on the bioactivities and the bioactive compounds present in shellfish Since global aquaculture of shellfis has increased in quantity and value according to the FAO statistics of 2006 [3]; the nutraceutical quality of shellfis and the potential for future development are worth investigating.

35.2 Chemical compositions

35.2.1 Proximate composition

American medium-sized oysters weigh 12 to 20 g [4] and Canadian oysters from British Columbia weigh 11.1 to 21.0 g [5]. The market size of Japanese oysters has a wider weight range of 6.2 to 20.2 [6]. Meanwhile, oyster (*Crassostrea gigas*) cultured for 7 to 8 months and sampled from 7 different coastal sites in Taiwan had an average fles weight of 1.18 to 1.73 g per oyster. Moisture averaged 85.0%, protein 8.61%, lipid 1.02%, glycogen 0.71%, and ash 1.92% [7]. A more recent study showed that the moisture content of raw-shucked oyster averaged 82.1% [8], similar to the previous findings Hard clams (*Meretrix lusoria*) consisted of 11.3 to 16.7% of fles [9] with an average of 12.7% [7], moisture contributed to 80.3 to 83.0%, protein 10.5 to 12.6%, lipid 0.3 to 3.9%, and ash 2.0 to 2.5% [9], similar to the finding of Jeng *et al.* [7] with a moisture 81.3%, protein 11.01%, lipid 0.83%, glycogen 0.64 %, and ash 3.08%.

35.2.2 Minerals

The main mineral component in ash contributes 1.81% to the wet weight (ww) of the oyster tissue [10]. The major elements present in oysters are Na, K, Ca, Mg, and metals such as Zn, Fe, and Cu within safety limits, in addition to trace amounts of Pb, Ni, Cd, and Hg [11]. Zn and Mn are high in cultured oysters sampled from southern China, followed by cultured *Pinctada martensii* Dunkeer, which is also high in selenium [8].

35.2.3 Extractive nitrogenous compounds

Total free amino acids contribute 0.91 to 1.30% in hard clams, while taurine alone contributes 39 to 56% to the total free amino acids, followed by alanine, glutamic acid, arginine, and glycine. These fi e amino acids account for 84 to 91% of the total free amino acids [9]. Alanine and glycine taste sweet and glutamic acid is the key umami compound in foods [12]. The principal taste-active components in scallop (*Patinopecten yessoensis*) are glutamic acid, glycine, alanine, arginine, adenosine 5′-monophosphate (AMP), Na^+, K^+, and Cl^- [13].

Univalves such as disk abalone (*Haliotis discus*) show a similar pattern of having high total free amino acids (1.96–3.82%). Taurine contributes 58 to 66% to the total amino acids during February to July and 34 to 48% between September and December [14]. The high content of free amino acids in clam tissue is responsible for the osmoregulation in saltwater bivalves and univalves. The high content of taurine is unique to shellfish

Freshwater clams contain much less free amino acids than the saltwater clams. The hot water extract of the freshwater clams marketed as clam essence shows that it has only 13% total free amino acids and 22% of the taurine content of hard clam fles [15].

The oligopeptides present in abalone are mainly glutamine + glutamic acid (101.5 mg %) followed by glycine (10.3 mg %), asparagine + aspartic acid (4.5 mg %), proline (4.1 mg %), and arginine (3.0 mg %) [16]. Oligopeptides consisting of glutamic acid, glycine, and/or alanine have fl vour potentiating activity [17]. The tripeptide, acorbine, β-Ala-Orn-Orn isolated from the Japanese clam (*Corbicula japonica*) is induced by cold-stress [18].

Adenosine 5′-triphosphate(ATP)-breakdown compounds are present in shellfish Among all the ATP derivatives, AMP is the highest in concentration at 25.26 mg/100 g with a K value averaged at 34.76% in freshly-shucked oyster, while those surveyed in commercial fresh oysters had 17.09 mg/100 g AMP and K values averaged 56.87% [10].

Trimethylamine oxide (TMAO) and trimethylamine (TMA) are also present in abundance, with a total of 8 to 17 mg/100 g in cultured hard clam [9]. TMAO is present in saltwater shellfis as an osmoregulator. During storage, the sweet-taste TMAO is reduced to TMA, which does not taste sweet. Other quaternary ammonium bases identifie in oyster meat and drip are glycine and betaine in much higher content than homarine trigonelline followed by TMAO [19,20].

35.2.4 Lipid and sterols

Lipids contribute to 2% of shellfis flesh In general, unsaturated fatty acids are higher than saturated acids in all tissues. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) accounts for 25.26 to 27.21% of the total lipids determined in 3 cultured bivalves, *Clamys nobilis*, *Perna viridis*, and *Pinctada matensii* [21]. In general, hepatopancreas has the highest lipid content (4.76–7.56% ww) while gonad has 1.34 to 1.70% lipid. The fles has 0.92 to 2.93% lipids. In some bivalve species, omega-3 (n-3 or ω-3) fatty acids can reach 45% of

total lipids [22]. Bivalves originating from temperate waters have a high DHA content, but not as high as that in marine fis from higher latitudes. Mussel (*Mytilus* spp) contains 114 to 226 mg/100 g DHA, surf clam (*Spisula* spp) 209 to 228 mg/100 g, quohog clam 373 to 390 mg/100 g, and American oysters 391 to 465 mg/100 g. In addition, the top shell-like moon snail (*Lunatia triseriata*) has 219 mg/100 g DHA. Since the development of the human central nervous system requires DHA and arachidonic acid, shellfis are considered as a good source of brain-specifi lipids [23].

Sterols are present in the unsaponifiabl fraction of lipid in clams. Thirteen sterols were found in muscle and viscera of marine bivalve *Megangulus zyonoensis* from the coastal waters of Hokkaido [24]. Cholesterol was the most abundant sterol followed by 24-methylenecholesterol. Muscle had significantl less brassicasterol than viscera, while campesterol and sitosterol were significantl higher in muscle than in viscera [24]. Epidioxysterols occur in hard clams [25], as 3,5,9-trihydroxyergost-7-en-6-one and 3,7,9-trihydroxycholest-5-ene [26], while campesterol and stigmasterol [27] occur in freshwater clams. Since clams cannot synthesize these phytosterols from acetate or from mevalonate [28,29], they are likely metabolized from the microalgae filtere into clams. Currently, freshwater clams and microalgae are being cultured, and feeding trials are ongoing in our laboratory to study the phytosterols metabolism in clams (unpublished).

35.3 Functional activities

35.3.1 Antioxidative activity

The hot-water extract of freshwater clams fed to an alternative animal model, tilapia (*Oreochromis mossambicus*), exhibited significantl ($P < 0.01$) increased total antioxidant capacity of blood plasma and prolongation of the lag phase was low-density lipoprotein (LDL) oxidation in human and tilapia plasma. The prolongation of the lag phase is dose-dependent ($r^2 = 0.98$), as shown by *in vitro* and *ex vivo* experiments (Fig. 35.1). The LDL oxidation rate in the propagation phase and the maximal oxidative state are reduced by the dietary intake of the clam extract [30].

35.3.2 Hypolipidemia and hypocholesterolemia activity

Total cholesterol, triacylglycerols (TAG), and LDL cholesterol were reduced in the plasma of tilapia fed feed fortifie with dried hot-water extract of freshwater clam (Table 35.1) [30], which is effective in cholesterol and lipid regulation.

In a current study on the Sprague Dawley rat fed freshwater clam hydrolysate for 4 weeks, TAG in plasma and liver is reduced by 66 and 18%, respectively, while plasma high-density lipoprotein (HDL) increases by 66%. Total cholesterol in the liver decreases by 50% and total cholesterol and bile acid secreted in the faeces increases by 1.8- and 1.2-fold, respectively [31,32]. It seems that dietary intake of freshwater clam extract or hydrosylate can enhance bile acid secretion and its binding of cholesterol lowers the plasma and liver cholesterol.

Plant sterols and their derivatives are the active compounds identifie in clams (Fig. 35.2), while plant stanol esters have been shown to be effective in cholesterol-lowering substances to young adults, elderly men, pre- and post-menopausal women, and children, with and without hypercholesterolemia, type-II diabetics, and coronary heart disease (CHD) risks, among others [33]. Therefore, shellfis can be considered a food source with potential cholesterol-lowering properties.

(a)

(b)
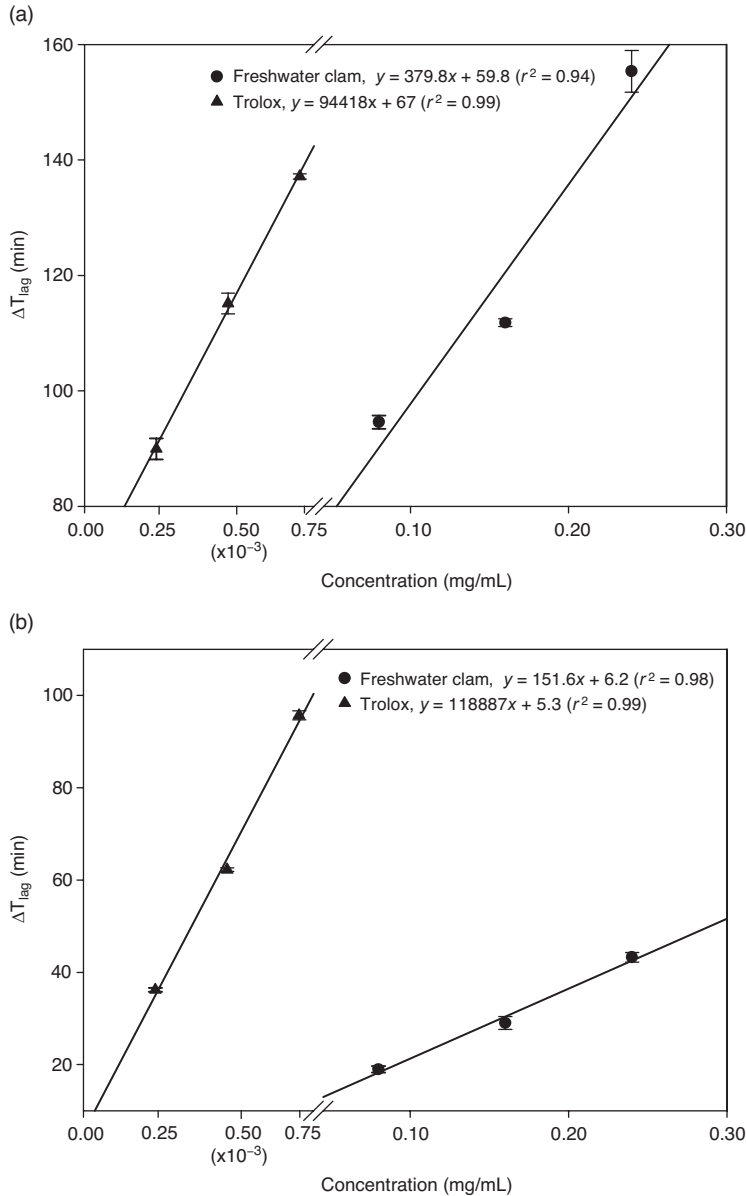
Fig. 35.1 Relationship between concentrations of hot-water clam extract (FC-HW) and prolongation of lag phase of (a) human (b) tilapia *in vitro* LDL oxidation. Adapted from Chen *et al.* [30], with permission of John Wiley & Sons, Inc.

35.3.3 Immunity regulation activity

The eight species of shellfish including freshwater clam (*Corbicula flumine*), oyster (*Crassostreas gigas*), hard clam (*Meretrix lusoria*), green mussel (*Perna viridis*), venus clam, hard clam (*Ruditapes philippinarum*), blood cockle (*Anadara granosa*), constricted tagelus

Table 35.1   Differences on blood lipids (mg/dL) of tilapia fed hot-water clam extract (FC-HW) for 60 days. Adapted from Chen *et al.* [30], with permission of John Wiley & Sons, Inc.

Plasma lipid	Control (mg/dL)	FC-HW (mg/dL)[a]
Total cholesterol	924 ± 271	353 ± 71.9*
LDL-cholesterol	570 ± 233	259 ± 56.7*
HDL-cholesterol	75 ± 5	66 ± 9
TAG	1397 ± 235	143 ± 46.9**

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein; TAG, triacylglycerols. The values are expressed as means ± standard deviation. Initial total cholesterol: 245 mg/dL and TAG: 114 mg/dL.
* $P < 0.05$, ** $P < 0.01$, significantly different from the control.
[a] 2% freshwater clam extract in feed (w/w).
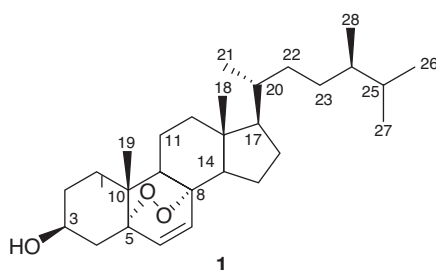
(1) 5α, 8α-epidioxy-24(R)-methylcholest-6-en-3β-ol
(2) 5α, 8α-epidioxy-24(S)-methylcholest-6-en-3β-ol
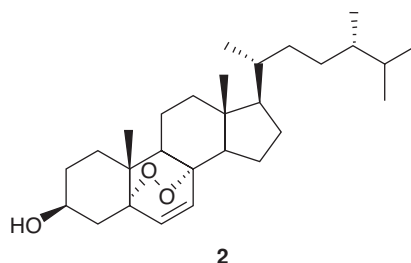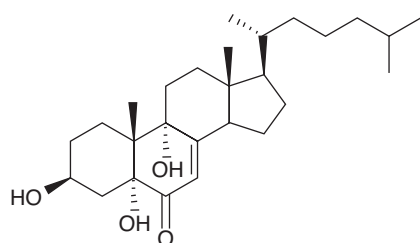
(3) 3,5,9-trihydroxy-ergost-7-en-6-one
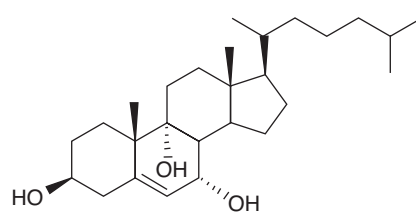
(4) 3,7,9-trihydroxy-cholest-5-ene
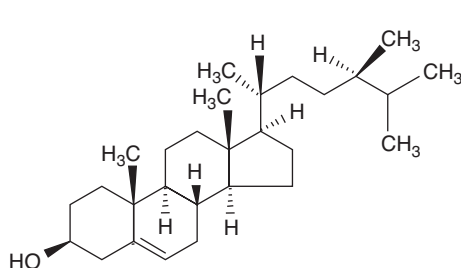
(5) Campesterol
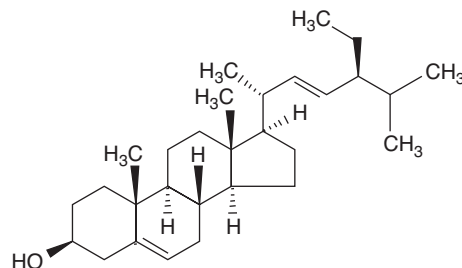
(6) Stigmasterol

Fig. 35.2   Structures of sterols isolated from clams. (1) and (2) epidioxysterols (EDS) isolated from *Meretrix lusoria* [25] and (3)–(6) isolated from *Corbicula fluminea* [26,27].

(*Sinonovacula constricta*), and honey cowrie (*Cypraea helvola*), were able to induce Immunoglobulin M (IgM) secretion when the individual shellfis extract was incubated with HB4C5 and SI102 hybridoma cells, respectively. The IgM secretion shows a dose-dependent manner when shellfis extract was treated with the hybridoma cells. A Concanavalin A-binding glycoprotein was found to be responsible for the induction of IgM secretion [34].

35.3.4 Anti-cancer activity

Shellfis extracts showed a dose-dependency of growth inhibition of hepatoblastoma (HuH-6KK) cells [34]. Two isomers (EDS) of apoptotic-inducing epidioxysterols (Fig. 35.2) were present in the ethyl acetate extract (EA) of hard clams (HC). The HC-EA and EDS inhibited cell proliferation with dose-dependency in human promyelocytic leukemia (HL-60) cells. EDS behaved as a potent inhibitor against cell viability, induced chromatin condensation, DNA fragmentation, and inhibited lipopolysaccharide(LPS)-stimulated nitric oxide (NO) generation in a dose-dependent manner [25].

The molecular mechanism of HC-EA involved inducing apoptosis. It includes disruption of mitochondrial cytochrome c into cytosol and subsequent activation of caspase 9 and caspase 3. HC-EA also causes HL-60 cells to rapidly lose glutathione and stimulates reactive oxygen species (ROS). Antioxidant enzymes, namely catalase and superoxide dismutase, significantl inhibit HC-EA-induced apoptosis [35]. A mechanism proposed for the induction of apoptosis by hard clam extract is shown in Fig. 35.3.

Freshwater clam extract showed similar anti-cancer effects and similar mechanisms as shown by the hard clam extract. Treatment of HL-60 cells with freshwater clam extract causes induction of caspase-2, caspase-3, caspase-6, caspase-8, and caspase-9 activities in a time-dependent manner. It also induced proteolysis of DNA fragmentation factor (DFF-45) and poly(ADP-ribose)polymerase (PARP). In addition, a rapid loss of mitochondrial transmembrane potential, generation of ROS, release of cytochrome c, and glutathione (GSH) depletion, explain the main apoptotic mechanism induced by freshwater clam extract [36].

35.3.5 Hepatoprotective activity

The hot-water extract of freshwater clams has been found to exert hepatoprotective effects on subacute hepatitis induced by carbon tetrachloride (CCl_4). Supplementation with 5 and 15% of the extract reduces CCl_4-induced liver injury as indicated by plasma glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), or alanine-amino transferase (ALT), and total cholesterol in Sprague Dawley rats [37]. The same extract also had a plasma lipid-lowering effect and reversed the elevated GOT and GPT in alcoholic liver injury in pig [38].

The trilogy of liver injury proceeds from normal liver to fatty liver due to the deposit of fat leading to liver enlargement, which is reversible by strict abstinence. The second stage is liver fibrosi due to the formation of scar tissue, which remains after recovery from liver injury. The third stage is cirrhosis caused by growth of connective tissues that destroys liver cells and becomes irreversible [39]. A current study using CCl_4 or dimethylnitrosamine to induce liver damage in the Sprague Dawley rat is ongoing [40]. The dietary intake of freshwater clam extract reduced liver organ weight, plasma GOT, GPT, fatty change, necrosis, bile duct proliferation-inflammation and fibrosi in rat liver.
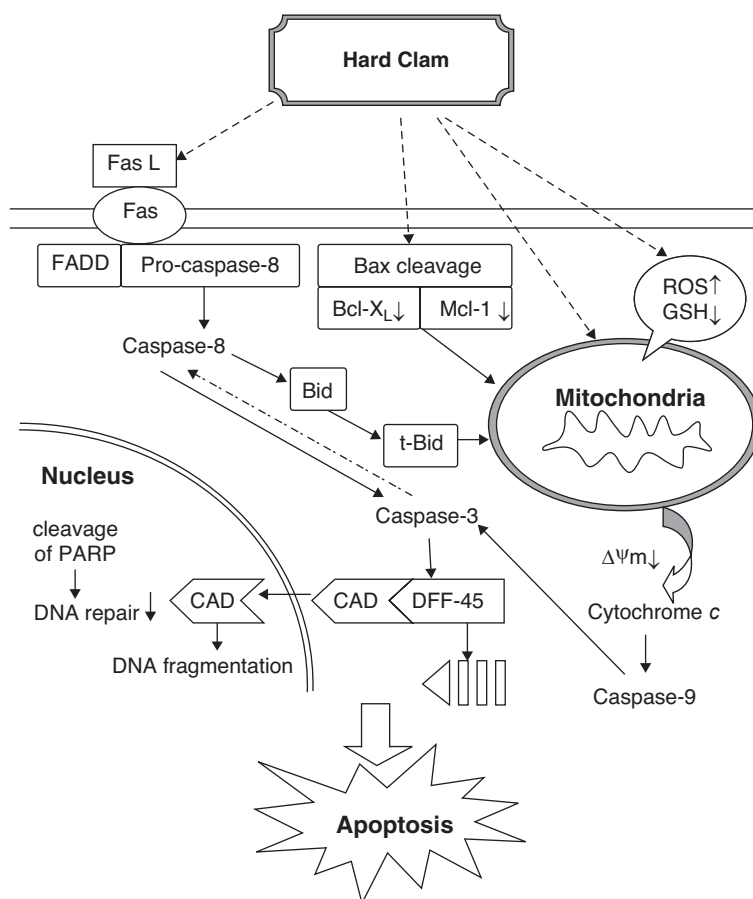
Fig. 35.3 Schematic representation of action mechanism by which HC-EA-induced apoptosis in HL-60 cells. Adapted from Pan *et al.* [35]. Copyright 2006, with permission of Elsevier.

35.4 Functional clam products

35.4.1 Clam essence

In Chinese traditional concept, clam is known to be liver-protective, but this belief lacks science-based evidence. Nevertheless, hot-water extracts of oyster (*Crassostrea gigas*), hard clam (*Meretrix lusoria*), and freshwater clam (*Corbicula flumine*) have been commercialized as essence or capsules. The annual market growth in 2008 for freshwater clam essence was 26% in Taiwan [41]. The product (certifie by the Health Department, No A00107) claims to lower the GOT (AST) and GPT (ALT) for those consuming the product.

35.4.2 Clam hydrolysates

Laboratory studies use proteases such as Protamex, Alcalase, Prozyme 6, Protease N, Protease A, or Flavourzyme, or a selected combination, to hydrolyze the residual clam meat after hot-water extraction of freshwater clam, hard clam, or oyster [31,42–45]. Antioxidative

activity and angiotensin I-converting enzyme (ACE) inhibitory activity were stronger in these hydrolysates than the unhydrolyzed hot-water extracts of the clams.

35.4.2.1   *Antioxidative activities*

The 80% ethanol extract of the freshwater clam hydrolysates inhibited linoleic acid peroxidation, scavenged α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals, and exhibited reducing power [42]. Similar antioxidative activities can also be found in the ethanolic extracts of hard clam or oyster. The short-chain peptides were found to be responsible for the observed antioxidative activities. The 50% inhibitory concentration (IC_{50}) values for inhibition of peroxidation and scavenging of free radicals were 1.89 to 2.29 mg/mL and 0.59 to 1.10 mg/mL, respectively for the hydrolysate of hard clam [43]. For the oyster hydrolysate, the IC_{50} values for inhibition of peroxidation and chelating Fe^{3+} are 0.97 to 1.25 mg/mL and 0.38 to 0.71 mg/mL, respectively [44]. Taurine and ornithine (orn) were two characteristic amino acids of shellfish Carnosine is a versatile antioxidative and anti-glycating agent implicated in neuroprotection [46].

35.4.2.2   *ACE-inhibitory activities*

The residual freshwater clam meat from the essence preparation can be hydrolyzed by Protomex (PX) for 5 hours followed by Flavouzyme hydrolysis for 0.5 hour. The hydrolysate is inhibitory to ACE at an IC_{50} of 0.043 mg/mL, while the positive control, captopril has an IC_{50} of 0.0015 µg/mL. The peptides (420~380 Da) responsible for the activities are Valine-Lysine-Proline (Val-Lys-Pro) (IC_{50} = 3.7 µM) and Valine-Lysine-Lysine (Val-Lys-Lys) (IC_{50} = 1,045 µM), while captopril has an IC_{50} of 0.0069 µM. All showed competitive inhibition. The highest inhibitory efficien y ratio is 1,314%/(mg/mL) for the 420~380 Da fraction. The PX digest of freshwater clam administered as a drink to spontaneously hypertensive rats (SHR), significantl reduced both systolic and diastolic blood pressure of the SHR [31]. Valine-Glutamine-Valine (Val-Glu-Val) from the peptide digest of short-necked clam and Leucine-Valine-Glutamine (Leu-Val-Glu) from the pearl oyster hydrolysate are ACE-inhibitory tripeptides with IC_{50} = 8.7 µM and 14.2 µM, respectively [47,48].

The hard clam hydrolysates also had ACE-inhibitory effects. The highest inhibitory activity was due to the fraction of 300 to 350 Da in molecular weight. The amino acid sequence is Tyrosine-Asparagine (Tyr-Asn), IC_{50} = 51 µM, and the inhibitory efficien y ratio is 5831%/(mg/mL) [45]. A hard clam (*Meretrix meretrix* Linnaeus) peptide (M_2) of molecular weight 18.4 kDa showed an inhibitory effect on proliferation of human gastric cancer cell BGC-823; the IC_{50} was 10 µg/mL. This M_2 activates alkaline phosphatase (ALP) and superoxide dismutase (SOD) and inhibited tyrosinase of which the IC_{50} was 1.5 µg/mL [8]. Short-necked clam (*Ruditapes philippinarum*) showed antitumour activity against HL-60 cells. The activity was contributed by glycosaminoglycan consisting of hexosamine, hexouronic acid, sulphate, fucose, and galactose [49].

35.5   **Conclusions**

Scientifi evidence has demonstrated that shellfis extracts and hydrolysates are effective and display antioxidative activity, lipidemic regulation, hypocholesterolemia, blood tension regulation, immunity regulation, and hepatic protection based on biochemical indices in

the cell model, and animal models including rats, hamsters, and pigs. Tilapia may also serve as an alternative animal model to demonstrate the nutraceutical effects of lipidemic regulation, hypocholesterolemia, and glycemia regulation, due to the fact that tilapia can easily be induced to hyperglycemia and hyperlipidemia. Shellfis products have been mainly marketed in Asia. With the global increases in population of metabolic syndrome, cancer and liver diseases, functional products derived from shellfis may serve as nutraceutical foods and chemopreventive agents for rendering health benefit for the entire population of the world.

35.6 Acknowledgements

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36 Marine oils and other marine nutraceuticals

Fereidoon Shahidi and Cesarettin Alasalvar

36.1 Introduction

Seafoods and marine products not only provide a wide range of fl vourful products, but also serve as an excellent source of a myriad of bioactives that render beneficia health effects and participate in disease risk reduction. Of particular interest are omega-3 (n-3 or ω-3) fatty acids that are found abundantly in marine foods as well as proteins, biopeptides, minerals, carotenoids and carotenoproteins, enzymes, and chitinous materials such as chitin, chitosan, chitosan oligomers, glucosamine, and other specialty products [1]. Table 36.1 provides a list of marine nutraceuticals and their application areas. The importance of nutraceuticals from aquatic resources is well recognized and this chapter provides a cursory account of selected bioactives from the marine environment.

36.2 Specialty and nutraceutical lipids

The occurrence and health benefit of long-chain omega-3 polyunsaturated fatty acids (PUFA) in seafoods and other marine organisms is a well established fact [2–4]. These fatty acids are produced in phytoplanktons in the oceans and are then consumed by fis and other marine species. Thus, n-3 PUFA may be procured from marine algae, body of fatty fish liver of white lean fish and the blubber of marine mammals. The constituent fatty acids present in such oils include eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3), and to a lesser extent docosapentaenoic acid (DPA, 22:5 n-3) in different proportions, depending on the species involved. In addition, liver oil from white lean fis serves as an excellent source of vitamin A, while that of shark has a high content of squalene and other bioactives.

The n-3 family of fatty acids is derived from the "parent" fatty acid α-linolenic acid (ALA, 18:3 n-3), which cannot be made by humans and must be provided in the diet and hence is considered as an essential fatty acid (EFA). Table 36.2 summarizes the fatty acid composition of selected marine and algal oils produced commercially. As can be seen, the contents of EPA, DHA, and DPA in each oil are dependent on the source material. Thus, the ratio of EPA to DHA in menhaden, cod liver, and seal blubber oils varies considerably, but the algal oil

Table 36.1 Nutraceuticals and bioactive ingredients from marine resources

Component	Application area
Omega-3 oils	Nutraceuticals, cardiovascular, immune response, inflammation, mental disorders, etc.
Chitin, chitosan, their oligomers	Dietary supplement, food, water & juice clarification,
Glucosamine	agriculture, & specialty products
Chondritin sulphate	Dietary supplements, arthritis, etc.
Squalene	Skin care, etc.
Biopeptides	Nutraceuticals, ACE inhibition, blood pressure, etc.
Carotenoids/carotenoproteins	Nutraceuticals, aquaculture, etc.
Enzymes	Processing application, specialty areas, etc.
Minerals (calcium)	Nutraceuticals
Shark cartilage, chondroitin sulphate, & squalene	Nutraceuticals
Others	Miscellaneous

Abbreviation: ACE, angiotensin converting enzyme.

tested almost exclusively contained DHA. Furthermore, positional distribution of n-3 fatty acids in such oils, again, depends on source material. The n-3 fatty acids are primarily located in the sn-2 position of triacylglycerols (TAG), while they are present mainly in the sn-1 and sn-3 positions of seal blubber oil.

Marine oils, similar to other edible oils, are subjected to different processing steps of refining bleaching, and deodorization (RBD). As protective components of oils are generally removed to a large extent during processing, it is important to treat the resultant RBD oils with appropriate antioxidants in order to enhance their oxidative stability. Microencapsulation provides another means for extending the shelf-life of highly unsaturated oils. Regardless, such oils increase the body demand for vitamin E. Therefore, addition of vitamin E, usually in the form of mixed tocopherols, to highly unsaturated oils is necessary for enhancing their oxidative stability and also to augment the body's need for vitamin E.

The use of marine oils containing n-3 fatty acids is recommended for foods that are used within a short period of time in order to avoid possible off-fl vour development during their expected shelf-life. While it is possible to mask some of the off-fl vours generated due to production of fl vour-active secondary oxidation products such as aldehydes, ketones, etc,

Table 36.2 Major fatty acids (weight %) of omega-3 rich marine and algal oils

Fatty acid	Menhaden	Cod liver	Seal blubber	Algal[a]
14:0	8.32	3.33	3.73	14.9
16:0	17.4	11.0	5.58	9.05
16:1 n-7	11.4	7.85	18.0	2.20
18:0	3.33	3.89	0.88	0.20
18:1 n-9, n-11	12.1	21.2	26.0	18.9
20:1 n-9	1.44	10.4	12.2	–
20:5 n-3	13.2	11.2	6.41	–
22:1 n-11	0.12	9.07	2.01	–
22:5 n-3	2.40	1.14	4.66	0.51
22:6 n-3	10.1	14.8	7.58	47.4

[a]Algal oil is DHASCO (docosahexaenoic acid single cell oil).

Table 36.3 Different food products in which omega-3 oils are incorporated

Class	Item
Dairy	Milk, yogurt, yogurt-based drinks, butter, & ice cream
Grain-based	Breads, cereal, pastas, crackers, & noodles
Confectionary	Sweets, candies, cakes, & bars
Spreads	Margarine & spreads
Dressings	Salad dressings, mayonnaise, & others
Juices	Orange juice & fruit juices
Meats & seafoods	Meat, fish, & poultry
Infant formulas	Milk, formulas, etc.
Others	Supplements, eggs, etc.

presence of primary products of oxidation remains a concern. Another way of introducing n-3 fatty acids into food products is to employ adequately microencapsulated products that may remain intact until they reach the gastrointestinal tract. In this way, there is no fl vour effect on the product even if the oil used initially contained some oxidation products. Table 36.3 summarizes a number of food products that are usually selected to enrich them with n-3 oils.

The role of marine lipids in health promotion and disease risk reduction with respect to the vascular system in humans has been well understood. The earlier work by Bang *et al*. [5] and Bjerregaard and Dyerberg [6] showed that, despite their high fat intake, Greenland Eskimos had a much lower incidence of myocardial infarction (MI) than their Dane counterparts. This was explained by the ratio of n-3 to n-6 fatty acid intake by these people (Table 36.4). However, the beneficia health effects of marine oils are manifold, and go beyond those related to coronary heart disease (CHD), visual and cognitive development, psychiatric conditions, inflammator diseases, Crohn's disease, and type-2 diabetes, among others. The n-3 fatty acids, especially DHA, are known to dominate the fatty acid profil of brain and retina lipids and play a major role in the development of the fetus and infants, as well as the health status and body requirements of pregnant and lactating women.

For therapeutic purposes, the natural sources of n-3 fatty acids as such may not provide the necessary amount of these fatty acids and hence production and use of n-3 concentrates may be required [7]. The n-3 concentrates may be produced in the free fatty acid (FFA), simple alkyl ester, and acylglycerol forms. To achieve this, physical, chemical, and enzymatic processes may be employed for concentrate production. The available methods suitable for this purpose, on an industrial scale, are low-temperature crystallization, fractional or molecular distillation, urea complexation, chromatography, supercritical flui extraction,

Table 36.4 Incidence of myocardial infarction (MI) and dietary lipid intake of Greenland Eskimos and Danes

Parameter	Eskimos	Danes
MI	3	40
Energy from lipids (%)	39	42
n-6 PUFA (g/day)	5	10
n-3 PUFA (g/day)	14	3
n-3/n-6	2.8	0.3
Cholesterol	790	420

and enzymatic splitting, etc. [8] (see Chapter 38 for detailed information). These procedures have been used, albeit to different extents, by the industry to prepare concentrates that are often sold in the ethyl ester form or re-esterifie with glycerol to be offered as TAG to the market. However, it has been demonstrated that acylglycerols are more stable than their corresponding ethyl esters. Regardless, the modifie oils need to be stabilized using synthetic or preferably natural antioxidants.

In preparation of modifie lipids containing n-3 fatty acids, structured lipids (SL) may be produced. SL are TAG or phospholipids (PL) containing combinations of short-chain, medium-chain, and long-chain fatty acids (SCFA, MCFA, and LCFA, respectively) located in the same molecule and may be produced by chemical or enzymatic means [9,10]. Structured lipids are developed to fully optimize the benefit of their fatty acid constituents in order to affect metabolic parameters such as immune function, nitrogen balance, and lipid clearance from the bloodstream. These specialty lipids may be produced via direct esterifica tion, acidolysis and hydrolysis, or inter-esterification We have used the acidolysis process to incorporate capric acid or lauric acid into seal blubber oil [11,12]. In addition, we produced 91% γ-linolenic acid (GLA, 18:3 n-6) concentrate from borage oil [13], which was subsequently used in acidolysis of menhaden and seal blubber oils [14]. Such structured lipids that include GLA, EPA, and DHA were also prepared using borage and evening primrose as a source of GLA and acidolysis with EPA and/or DHA [15,16]. The products so obtained, while similar to those produced by incorporation of GLA into marine oils, differ in the composition and distribution of fatty acids involved.

36.3 Bioactive peptides and proteins from marine resources

Hydrolysis of the amide linkage in the protein chain leads to the formation of peptides with different numbers of amino acids as well as free amino acids. While enzymes with endopeptidase activity provide peptides with different chain lengths, exopeptidases liberate amino acids from the terminal positions of the protein molecules. Depending on reaction variables as well as the type of enzyme, the degree of hydrolysis of proteins may differ considerably. The peptides produced from the action of a specifi enzyme may be subjected to further hydrolysis by other enzymes. Thus, use of an enzyme mixture or several enzymes in a sequential manner may be advantageous. The peptides so obtained may be subjected to chromatographic separation and then evaluated for their amino acid sequence as well as their antioxidant and other activities.

In a study on capelin protein hydrolysates, four peptide fractions were separated using Sephadex G-10. While one fraction exerted a strong antioxidant activity in a β-carotene/starch linoleate model system, two fractions possessed a weak antioxidant activity and the fourth one had a prooxidant effect. Two-dimensional high-performance liquid chromatography (HPLC) separations showed spots with both pro- and antioxidant effects [17]. Meanwhile, protein hydrolysates prepared from seal meat were found to serve as phosphate alternatives in processed meat applications and reduced the cooking loss considerably [18]. Furthermore, Alaska pollock skin hydrolysate was prepared using a multienzyme system in a sequential manner. The enzymes used were in the order of Alcalase, Pronase E, and collagenase. The fraction from the second step, which was hydrolyzed by Pronase E, was composed of peptides ranging from 1.5 to 4.5 kDa and showed a high antioxidant activity. Two peptides were isolated (Table 36.5), using a combination of chromatographic procedures, and these

Table 36.5 Antioxidative peptides from Alaska pollock skin hydrolyzate and Soy 7S Protein. Adapted from Kim *et al*. [19] and Chen *et al*. [20]

Peptide	Amino acid sequence
Alaska Pollock	
P_1	Gly-Glu-Hyp (Gly-Pro-Hyp)$_3$-Gly
P_2	(Gly-Pro-Hyp)$_4$-Gly
Soy 75 Protein	
P_1	Val-Asn-Pro-His-Asp-His-Glu-As
P_2	Leu-Val-Asn-Pro-His-Asn-His-Glu-Asn
P_3	Leu-Leu-Pro-His-His
P_4	Leu-Leu-Pro-His-His-Ala-Asp-Ala-Asp-Tyr
P_5	Val-Ile-Pro-Ala-Gly-Tyr-Pro
P_6	Leu-Gly-Ser-Gly-Asp-Ala-Leu-Arg-Val-Pro-Ser-Gly-Thr-Tyr-Tyr

were composed of 13 and 16 amino acid residues [19]. The sequence of the peptides involved is also given in Table 36.5 and compared with those of soy 7S protein hydrolysates [20]. These peptides exert their antioxidant activity via free radical scavenging as well as chelation effects. Recently, proteases from shrimp processing discards were characterized [21] and application of salt-fermented shrimp by-product sauce as a meat tenderizer was reported [22].

36.4 Chitin, chitosan, chitosan oligomers, and glucosamine

Chitin is recovered from processing discards of shrimp, crab, lobster, and crayfis following deproteinization and demineralization [23]. The chitin so obtained may then be deacetylated to afford chitosan [23]. Depending on the duration of the deacylation process, the chitosan produced may assume different viscosities and molecular weights. The chitosans produced are soluble in weak acid solutions, thus chitosan ascorbate, chitosan acetate, chitosan lactate, and chitosan malate, etc., may be obtained and these are all soluble in water. Chitosan has a variety of health benefit and may be employed in a number of nutraceutical and health-related applications. Chitosan derivatives may also be produced in order to obtain more effective products for certain applications.

Chitosans with different viscosities were prepared and used in an experiment designed to protect both raw and cooked fis against oxidation as well as microbial spoilage [24–26]. The content of propanal, an indicator of oxidation of n-3 fatty acids, was decreased when chitosan was used as an edible invisible fil   in herring. Furthermore, the effects were more pronounced as the molecular weight of the chitosan increased. In addition, inhibitory effects of chitosan coatings in the total microbial counts for cod and herring showed an approximate 1.5 and 2.0 log cycles difference between coated and uncoated samples, respectively, after 10 days of refrigerated storage (results not shown). However, to have the products solubilized in water without the use of acids, enzymatic processes may be carried out to produce chitosan oligomers. Due to their solubility in water, chitosan oligomers serve best in rendering their benefit under normal physiological conditions and in foods with neutral pH. Furthermore, depending on the type of enzyme employed, chitosan oligomers with specifi chain lengths may be produced for certain applications [27].

The low-molecular-weight chitin and chitosan oligomers (also known as chitin/chitosan oligosaccharides (COSs) have received considerable attention as physiologically functional

Table 36.6 Antibacterial activity of different molecular weight chitosan oligomer (COS) fractions

Bacteria	Antibacterial activity (%)[a]		
	HMWCOSs[b]	MMWCOSs[c]	LMWCOSs[d]
Escheria coli[e]	98 ± 0	62 ± 6	51 ± 7
Escheria coli O-157[e]	71 ± 3	56 ± 4	60 ± 2
Salmonella typhi[e]	91 ± 2	88 ± 0	89 ± 0
Pseudomonas aeruginosa[e]	47 ± 5	35 ± 5	22 ± 8
Streptococcus mutans[f]	100 ± 0	99 ± 0	99 ± 0
Straphylococcus aureus[f]	97 ± 3	95 ± 0	93 ± 9
Straphylococcus epidermidis[f]	82 ± 0	57 ± 3	23 ± 1
Bacillus subtilis[f]	63 ± 5	60 ± 5	63 ± 7
Micrococcus luteus[f]	70 ± 0	67 ± 3	63 ± 7

[a]Following the incubation of bacterial culture with 0.1% different COSs fractions, the number of colonies formed on the medium was calculated as a percentage compared to the control.
[b]High molecular weight chitosan oligosaccharides (molecular weight range 10-5 kDa).
[c]Medium molecular weight chitosan oligosaccharides (molecular weight range 5-1 kDa).
[d]Low molecular weight chitosan oligosaccharides (molecular weight below than 1 kDa).
[e]Gram-negative.
[f]Gram-positive.

materials having antitumour, immuno-enhancing, and antibacterial activities [24,28,29]. Production of COSs via the hydrolysis of chitosan may be achieved chemically or enzymatically. The enzymatic production is preferred, but cost of enzyme may be prohibitive. Therefore, a continuous low-cost production method to produce COSs with desired molecular size has been developed [30]. The COSs (Table 36.6) with low-molecular-weight and hetero-chitosan oligosaccharides, have been reported to have antibacterial, radical scavenging, angiotensin converting enzyme (ACE) inhibitory, and anticoagulant activities [31]. The monomer of chitin, *N*-acetylglucosamine (NAG), has been shown to possess anti-inflammator properties. Meanwhile, glucosamine, the monomer of chitosan, prepared via HCl hydrolysis, is marketed as glucosamine sulphate. This formulation is prepared by addition of ferrous sulphate to the preparation. Glucosamine products may also be sold in formulation containing chondroitin 4- and chondroitin 6-sulphates. While glucosamine helps to form proteoglycans that sit within the space in the cartilage, chondroitin sulphate acts like a liquid magnet. Thus glucosamine and chondroitin work in a complementary manner to improve the health of the joint cartilage.

The by-products in the chitin extraction process from shellfis include carotenoids/ carotenoproteins and enzymes [32–35]. These components may also be isolated for further utilization in a variety of applications.

36.5 Enzymes

The aquatic environment contains a wide variety of genetic material and hence represents exciting potential for discovering different enzymes [36]. Therefore, much effort has been made to recover and characterize enzymes from fis and aquatic invertebrates [32]. Digestive proteolytic enzymes from stomachless marine fis such as conner, crayfish and puffer appear

to inactivate polyphenol oxidize and/or pectin esterase in fruit juices. Successful application of such enzymes has also allowed inactivation of polyphenol oxidase in shrimp processing as an alternate to sulphating [33]. Alkaline phosphates from shrimp may be used in different kits and some enzymes may be recovered and used in deskinning of fis and squid or cleaning of fis roe for caviar production, etc.

Decade ago, Haard and Simpson [37] edited a comprehensive book on seafood enzymes. Several marine enzymes are now used commercially in the food industry and some can be isolated as by-products of the seafood processing industry [38,39]. Enzymes from marine resources are likely to establish a significan part of the industrial enzymes market in future as we learn more about unique properties that fulfi the needs for specifi applications such as a nutraceutical ingredient [38].

Although there are many commercially available enzymes isolated from marine resources [37], most of these are used as biochemical research reagents. The use of marine enzymes as nutraceutical ingredients in food products are new and a growing area [39]. Most of the seafood enzymes used in the food industry are either for processing or preservation. The main use is in dairy technology. The major use of enzymes is in the coagulation of milk to make cheese [39]. However, other applications have been suggested such as meat tenderizing using specifi fis collagenases and the enzymatic clarificatio of fruit juice [40]. Arctic scallop [41] contains a cold-active peptide enzyme with lysozyme-like activity that is active against both gram-positive and gram-negative bacteria. Although this enzyme has potential as a nutraceutical ingredient in food products, it will probably be necessary to produce it by rDNA technology because it is present naturally in scallops only at a very low concentration [42]. The enzyme has been recovered from scallop processing wastes and has potential for application as a preservative in refrigerated foods [38].

36.6 Carotenoids

Carotenoids and carotenoproteins are present in salmonoid fis as well as in shellfish These carotenoids may be recovered from processing by-products and used in a variety of applications [35]. In addition, certain carotenoids, such as fucoxanthin occur naturally in seaweeds [43,44]. Fucoxanthin has been shown to have anti-proliferative activity on tumour cells and has also been implicated in having anti-obesity and anti-inflammator effects. Fucoxanthinol is a known metabolite of fucoxanthin [45,46].

36.7 Minerals and calcium

Among fis processing by-products, fis bone or skeleton serves as a potential source of minerals and calcium, the latter being an essential element for human health. Calcium from fis would be easily absorbed by the body [47]. However, to incorporate fis bone into calcium-fortifie foods, it is necessary to firs convert it into an edible form by softening its structure. This could be achieved by hot-water treatment and heat treatment in an acetic acid solution. Pepsin-assisted degradation of Alaska pollock bone in acetic acid solution led to the highest degree of hydrolysis and dissolution of both mineral and organic parts of fis bone [48,49]. As reported by Larsen *et al*. [47], the intake of small fis with bones could increase calcium bioavailability. Fish bone contains hydroxyapatite that, unlike other calcium

phosphates, does not break under physiological conditions and takes part in bone bonding. This property has been exploited for rapid bone repair after major trauma or surgery.

36.8   Shark cartilage, chondroitin sulphate, and squalene

Shark cartilage, extracted from the fin and heads of sharks, is a type of fl xible connective tissue found in the animal's skeletal system. Traditionally, shark cartilage has been eaten as a food and health supplement in the Far East. The market for shark cartilage has seen dramatic increases in recent years for its perceived anti-angiogenic properties in cancer therapy [50–53]. Commercial shark cartilage supplements are primarily composed of chondroitin sulphate, a key component in human cartilage that is essential for joint health. Shark cartilage is believed to help a variety of conditions, including arthritis, shingles, rheumatism, haemorrhoids, psoriasis, inflammator disorders, and anti-cancer properties.

Chondroitin sulphate, a bioactive component of shark cartilage, is a typical mucopolysaccharide sulphate [54]. It is mostly used as a base for cosmetics such as hand creams [55]. Chondroitin sulphate caused the reduction of body and parametrial adipose tissue weight and prevention of fatty liver and hyperlipidemia in mice fed a high-fat diet [56].

Ackman [57] pointed out that Greenland shark liver oil exhibited bioactivity against cancer. Shark liver oil is also known to contain a high proportion of squalene, up to 50% [57]. In other words, squalene is one of the main components of shark liver oil. Squalene is used in cosmetics as a skin softener and lubricant since low levels are found in our normal skin oils. It circulates in our bloodstream at low levels all the time and may also serve as a protecting agent against photooxidative damage. In addition, squalene acts as an antioxidant, oxygenates the system, helps boost immune function, and facilitates detoxificatio [57]. Squalene has been promoted as having cell-protecting abilities, which may reduce the side-effects of chemotherapy. Squalene is a good antioxidant under frying conditions for oils that contain it, such as olive oil.

36.9   Other nutraceuticals from marine resources

Seaweeds are a rich source of iodine, phlorotannins, glutathione, fucoxanthin, and also carbohydrates such as alginates. Much interest has also been expressed in algae because epidemiological evidence has linked habitual consumption of seaweed to reduced risk of a number of chronic diseases in Japanese and Chinese populations. Fish skin may also be used for production of collagen as well as gelatin, the latter for special nutritional, religious, and nutraceutical purposes. Meanwhile, unexploited aquatic resources from the oceans offer many opportunities for future developments [58,59].

36.10   Conclusions

Marine resources provide a rich reservoir of nutraceuticals and functional food ingredients. These ingredients belong to a wide range of chemical compounds with beneficia health effects. Therefore, their use in food and as natural health products, as well as in cosmetics, may be recommended.

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37 Nutraceuticals and bioactives from marine algae

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37.1 Introduction

Marine algae can be prokaryotic or eukaryotic and, in evolutionary terms, recent or very ancient. This diversity makes marine algae a potentially rich source of a wide array of bioactive components with applications in the food, nutraceutical, pharmaceutical, feed, aquaculture, cosmetic, fertilizer, and even in the fuel industries (e.g. natural gas, ethanol, and biodiesel, etc.). Most algae are phototrophic and require light as the free-energy source for their growth. However, some species of algae are capable of heterotrophic growth and do not need light as a free-energy source, but obtain metabolic energy from the dissimilation of organic carbon compounds. Dinoflagellate are early eukaryotic marine algae that can be phototrophic and/or heterotrophic and form an important part of the marine plankton. There is a wide range of cultivation techniques used in order to optimize growth and production ranging from the alteration of nutrient source, shifts in temperature, pH, dissolved oxygen, salinity, aeration quality, aeration rate, and individual nutrients. In a scale-up process, relatively high productivities (high biomass concentration per unit volume) can be obtained within 1 to 2 weeks. In such ways as those described, algae can be cultured to produce significan quantities of nutritionally important polyunsaturated fatty acids (PUFA) for extraction and purificatio at an industrial scale (Table 37.1). Docosahexaenoic acid (DHA; 22:6 n-3) is a characteristic omega-3 (n-3 or ω-3) PUFA of the marine algae. For nutraceutical applications, the PUFA may also be administered in the form of biomass. It has been surprisingly found that considerable quantities of PUFA can be obtained by culturing microalgae from the classes Dinoflagellate and Thraustochytrids, and subjecting the cultures to extraction with an organic solvent and subsequent purification *Crypthecodinium cohnii* is a unique heterotropic marine dinoflagellate in that DHA is almost exclusively the only PUFA present in its lipid [1] *Schizochytrium* spp. is also a heterotropic microalgae belonging to the Order Thrausochytriales with the Phylum Heterokonta, which can yield about 40% of DHA from its total fatty acid production. Successful cultivation of *Crypthecodinium cohnii* and *Schizochytrium* spp. to produce commercial algal oils containing DHA has been achieved commercially.

Marine algae can be divided into two groups of microalgae and macroalgae (seaweed). Microalgae are small microscopic aquatic photosynthetic plants. On the other hand, seaweeds

Table 37.1 Distribution of PUFA in marine algae

Group	Genus/species	PUFA
Dinoflagellates	*Crypthecodinium cohnii*	DHA
Thraustochytrids	*Schizochytrium sp.*	DHA/DPA
Thraustochytrids	*Ulkenia sp.*	DHA/DPA
Dinoflagellates	*Amphidinium sp.*	DHA
Red Algae	*Porphyridium*	ARA
Eustigmatophytes	*Nannochloropsis*	EPA
Phytoflagellates	*Isochrysis galbana*	EPA
Cyanobacteria (Blue-green algae)	*Spirulina sp.*	GLA

Abbreviations: PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid.

are large aquatic photosynthetic plants that can be seen without the aid of a microscope. Marine seaweeds of interest as food include Irish moss, laver, and kelp, which are eaten to some extent in different communities and serve as a mineral supplement in animal feed. Seaweeds are an important food source in many Asian countries such as Japan, China, and Korea, etc. Japanese cuisine employs different varieties (e.g. kombu, laver, and nori) for several uses including soups, vegetables, tea, sushi, and as a general seasoning. Seaweeds are a rich source of iodine and an important nutrient source. They also provide alginic acid, a jellylike substance that is used as a stabilizer and thickener in a wide variety of commercially processed foods such as ice creams, puddings, fl voured dairy beverages, pie fillings soups, and syrups, etc. [2–5].

Algae have an advantage over many other organisms in that they can be appropriately grown for the production of desirable bioactive compounds. The search for nutraceuticals and bioactive compounds from algae is steadily increasing as the increased number of species being brought into culture. The purpose of the present chapter is to survey the different types of bioactive chemicals encountered in marine algae, and to focus on microalgae as a commercially viable source for production of n-3 oils.

37.2 Carotenoids

Carotenoids belong to the category of tetraterpenoids. Several species of microalgae, especially green algae, accumulate high concentrations of carotenoids such as β-carotene, astaxanthin, and canthaxanthin. These carotenoids have wide applications as natural colorants and antioxidants. β-Carotene was the firs of these carotenoids to be commercialized from the biflagellate alga, *Dunaliella salina*. This alga occurs in a wide range of marine habitats such as oceans, brine lakes, and salt-water ditches near the sea, predominantly in water bodies containing more than 2 M salt and a high concentration of magnesium [6]. The ability to grow at very high salt concentrations, where few other organisms can survive, its high temperature tolerance (from −5°C to above 40°C), and the high cell content of β-carotene (up to 14% of dry weight) has made this alga an attractive candidate for commercial production of carotenoids. The extreme conditions under which this alga grows means that relatively simple cultivation systems can be used. Australia is the major producer of natural β-carotene from *Dunaliella*. The two commercial production plants in Australia use

Table 37.2   Secondary carotenoids (as a % of total carotenoids) composition of selected algae

Algal species	Astaxanthin monoester	Astaxanthin diesters	Free astaxanthin	Lutein/ zeaxanthin	β-Carotene	Reference
Haematococcus pluviali	74	10	–	9	–	[8,22]
Haematococcus spp.	70	25	5	–	–	[23]
Oocystis minuta	51	36	5	3	2	[8]
Euglena sanguinea	–	91	–	1	–	[24]
Crucigenella rectangularis	61	24	2	2	8	[8]
Muriellopsis sphaericum	62	28	2	5	2	[8]

large shallow ponds of several hundred acres in area to grow the alga. These ponds have a depth of 30 to 60 cm and are only mixed by wind and thermal convection. The harvested biomass is extracted and pure β-carotene or mixed carotenoids are sold as a nutritional supplement and natural food colorant. The dried β-carotene-rich *Dunaliella* powder is also sold as a feed additive for aquaculture to pigmented crustaceans such as prawns [6].

Another carotenoid of great interest is astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione). It was firs described in aquatic crustaceans (lobsters, crabs, and shrimps) as an oxidized form of β-carotene, which gives the marine invertebrates and fis the distinctive orange-red colour. Astaxanthin is used as a pigment for farmed salmonid fis as well as a dietary antioxidant. The astaxanthin molecule has two asymmetric carbons located at the 3 and 3′ positions of the benzenoid rings on either end of the molecule. The astaxanthin accumulated in microalgae occurs as the 3S and 3′S isomers in the form of mono- and diesters [7]. The content and composition of secondary carotenoids are different among various species of algae (Table 37.2).

The unicellular green alga, *Haematococcus*, has been reported as a promising micro-organism for commercial production of astaxanthin. *Haematococcus* produces chlorophylls *a* and *b*, and primary carotenoid compounds, namely β-carotene, lutein, zeaxanthin, violaxanthin, and neoxanthin. Astaxanthin exists in *Haematococcus* cells in the forms of mono- and diesters, which account for up to 95% of total secondary carotenoids [7]. *Haematococcus* can accumulate up to 6 to 8% astaxanthin in the biomass [8].

The production of algal astaxanthin from *Haematococcus pluvialis* is quite a different process from that used for β-carotene production from *Dunaliella salina*. The chlorophyte, *Haematococcus pluvialis* is a freshwater alga, which is normally grown in temporary water bodies such as depressions in rock, puddles, fl werpots, and birdbaths. The optimum temperature for growth is about 22 to 25°C. Furthermore, astaxanthin is produced in a thick-walled resting stage, the aplanospore, whereas maximum growth occurs in a green thin-walled flagellate stage. This necessitates a two-stage culture process, one optimized for biomass production and the other for astaxanthin production. Being a freshwater alga, open air culture as used for *Dunaliella*, *Chlorella*, and *Spirulina* is not feasible, and *Haematococcus* must be grown in a closed photobioreactor to avoid contamination. The large-scale culture systems proposed for this alga involve a growth stage in a closed, temperature controlled

photobioreactor to achieve maximum biomass, followed by an astaxanthin-accumulating stage under high light conditions, preferably in nutrient-poor medium. Commercial production is underway in the US and Israel, and other ventures have been proposed elsewhere. Cultivation methods have been developed to produce *Haematococcus* containing 1.5 to 3.0% astaxanthin by dry weight. It has applications as a pigment source in feeds and in the nutraceutical market. Dried astaxanthin-rich algal meal can also be pressed into tablets. However, the astaxanthin in these tablets is degraded easily by oxidation. Manufacturers of astaxanthin have attempted to suspend *Haematococcus* biomass in edible oils, anticipating that the oil would create a barrier between atmospheric oxygen and the astaxanthin-rich biomass [9]. Alternatively, supercritical flui extraction technology can be used to produce astaxanthin-rich oleoresins, which are then diluted with edible oils to the desirable concentration for microencapsulation. Research on algal production of other carotenoids such as lutein and canthaxanthin from other algae such as *Chlorella* spp. and *Chlamydomonas* spp. is underway.

37.3   Phycobilins

Phycobilins are complex photoreceptor pigments found in cyanobacteria and in the chloroplasts of Rhodophyta (the red algae), glaucophytes, and in those of Cryptophyceae, a class of biflagellat unicellular eukaryotic algae. There are two classes of phycobilins and they occur only in Cyanobacteria and Rhodophyta. The bluish pigment phycocyanin is found in Cyanobacteria and gives them their common name of "blue-green algae". The reddish pigment phycoerythrin is found only in Rhodophyta and gives them their common name of "red algae" (Table 37.3). In cyanobacterial cells and red algal chloroplasts, phycobiliproteins

Table 37.3   Distribution of chromophores in marine algae

Group	Occurrence	Chromophores	Colour
Cyanobacteria	Widespread	Chlorophyll *a*, Chlorophyll *d*, Phycocyanin, Phycoerythrin, Zeaxanthin, and Fucoxanthin	Blue-green
Rhodophyta	Marine	Chlorophyll *a*, Chlorophyll *d*, α-Carotene, β-Carotene, Xanthophylls, and Phycoerythrin	Red
Chlorophyta	Marine and fresh water	Chlorophyll *a*, Chlorophyll *b*, Lutein, Neoxanthin, and Violaxanthin	Green or orange
Bacillariophyta	Marine and fresh water	Chlorophyll *a*, Chlorophyll *c*, α-Carotene, β-Carotene, Fucoxanthin, and Diadinoxanthin	Yellow-green or brown
Chrysophyta	Marine and fresh water	Chlorophyll *a*, Chlorophyll *c*, Fucoxanthin, and Neoxanthin	Golden brown
Dinophyta	Marine and fresh water	Chlorophyll *a*, Chlorophyll *c*, Dinoxanthin, Peridinin, and Diadinoxanthin	Red or brown
Xanthophyta	Fresh water	Chlorophyll *c*, β-Carotene, Neoxanthin, and Violaxanthin	Yellow-green
Phaeophyta	Marine	Chlorophyll *a*, Chlorophyll *c*, Fucoxanthin, Violaxanthin, and Lutein	Olive-green to brown

are aggregated in highly ordered protein complexes called phycobilisomes, making these phycobilins unique among photosynthetic pigments. Phycobilisomes are attached to the cytosol (stromal) face of the thylakoid. Extending into the cytosol, the phycobilisomes consist of a cluster of phycobilin pigments including phycocyanin and phycoerythrin attached *via* thiol linkages to the associated protein. These particles serve as light-energy antennae for photosynthesis [10]. Phycoerythrin is associated with chlorophyll in the Rhodophyta, and enables them to be photosynthetically efficien in deep water where blue light predominates. The longer wavelength red portion of the spectrum that activate green chlorophyll pigments do not penetrate the deeper water of the photic zone, so green algae cannot survive at depth where red algae thrive.

The phycobilin pigments have applications as food colourings, in cosmetics, as fluorescen dyes for fl w cytometry, and in immunological assays. The main commercial sources are the Cyanobacterium *Spirulina* and the red unicells *Porphyridium* and *Rhodella*. Phycobilins have also found use as diagnostic indicators and research tools. Both pycocyanin and phycoerythrin fluoresc at a particular wavelength. That is, when they are exposed to strong light, they absorb the light energy and release it by emitting light of a very narrow range of wavelengths. The light produced by this fluorescenc is so distinctive and reliable that phycobilins may be used as chemical tags. The pigments are chemically bonded to antibodies, which are then put into a solution of cells. When the solution is sprayed as a stream of fin droplets past a laser and computer sensor, a machine can identify whether the cells in the droplets have been tagged by the antibodies. This has found extensive use in cancer research, for tagging tumour cells. The phycobilins are often used in research as chemical tags, for example, by binding phycobiliproteins to antibodies in a technique known as immunofluorescenc [10].

37.4   Polysaccharides

Marine algae are important sources of polysaccharides. Algae polysaccharides such as carageenan, alginate, fucoidan, and agar-agar have been of industrial importance, especially in the food industry. Different groups of algae are recognized as green algae (Chlorophycea), red algae (Rhodophycea), and brown algae (Phaeophycea); only the last two are used on an industrial scale. Of particular interest are the red algae as a source of various biochemicals, especially sulphated polysaccharides. These sulphated polysaccharides can be used as gelling agents, thickeners, stabilizers, and emulsifier in various food products. The well-known examples of the sulphated polysaccharides are agar-agar and carrageenan. The conventional sources of sulphated polysaccharides are red algae, which are usually harvested from their natural habitats. Many different polysaccharides may be extracted, but alginates and carrageenans are the more developed, especially as gelling agents; fucoidans are also under investigation due to the importance of sulphated polysaccharides for biological applications.

Alginates are cell-wall constituents of brown algae (*Phaeophycota*). Some 32,000 to 39,000 metric tones of alginic acid are annually extracted worldwide. The main producers are Scotland, Norway, China, and the US, with smaller production amounts by Japan, Chile, and France. Alginate has often been used for encapsulation of pharmaceuticals, bacteria, or yeasts in biotechnological processes.

Carrageenan is a powder extracted from various species of red algae that are farmed and processed. In many food applications, carrageenan is used as a suspending and emulsifying stabilizer, thickener, binder, and gelling agent. The outstanding properties of this product make it a very versatile ingredient. In meat and poultry products, it can be used as a fat replacer.

In non-food industries, this natural ingredient find more applications in pharmaceuticals and cosmetics.

Fucoidan is a polysaccharide that is rich in fucose and found mainly in brown algae. Fucoidan is beginning to emerge as a powerful tool for enhancing immunity and other important aspects of overall health and human well-being. Nutraceutical products containing purifie concentrates of U-fucoidan and F-fucoidan are currently being made available as an immune enhancing food supplement. In a number of *in vitro* and animal studies, it has inhibited coated viruses such as herpes, HIV, and human cytomegalovirus, a type of herpes virus that can cause blindness and fatal pneumonia in individuals with compromised immune systems. Studies have suggested that fucoidan may not only inhibit the initial stages of viral infection, such as attachment to and penetration into host cells, but also the later replication stages after virus penetration [11]. Fucoidan has also been shown to possess significan cardioprotective activity that may be of particular benefi to anyone with cardiovascular health conditions and/or for prevention of heart and blood vessel problems [12].

Carbohydrates of marine algae can be found in the form of sugar, starch, cellulose, and other polysaccharides. Of interest in this context are polysaccharides produced by some species of red algae. The cells of the red algae are encapsulated within a sulphated polysaccharide in the form of a gel. During growth in a liquid medium, the viscosity of the medium increased due to the dissolution of soluble polysaccharide from the cell surface into the medium. Knowledge of the chemical composition and structure of polysaccharides derived from the red microalgae is limited, due to their complexity and lack of specifi enzymes that degrade them. However, it is known that the polysaccharides of the different species of red algae are heteropolymers, having different chemical compositions and varying amounts of sulphate [13]. In all species studied, glucose, xylose, and galactose are found to be the predominant sugars of the polymers. In addition, small amounts of rhamnose, ribose, arabinose, and mannose have been detected. Algae-derived polysaccharides are negatively charged due to the presence of glucouronic acid and half-sulphate ester groups. The molecular mass of the polysaccharides of various species of red microalgae has been estimated to be 2 to 7×10^6 Da [14]. Algal polysaccharides also contain protein.

Algal polysaccharides may fin application in human and animal health as dietary fibr and antiviral agents. Studies have shown that serum cholesterols, triacylglycerols (TAG), and very low-density lipoprotein (VLDL) levels were considerably lower in rodents fed with *R. reticulata* biomass or its polysaccharides as compared to the control animals. In glucose loading experiments, the levels of serum insulin and glucose were much lower in rats fed with algal biomass or polysaccharide than in control animals [15]. Polysaccharides of marine algae have shown promising antiviral activity against *Herpes simplex* virus types 1 and 2 and *Varicella zoster* virus with no cytotoxic effects [16]. Polysaccharides of marine algae have also been found to be potent *in vitro* inhibitors of HIV [17].

37.5 Omega-3 oils

Interest in the nutritional importance of n-3 PUFA has increased markedly during the past decade. At present, selected fis oils and microalgal species are the main industrial sources of n-3 PUFA. However, the supply of fis oils may be unreliable due to the failure or variability of various fisheries Furthermore, there is concern that the supply of fis oils will be limited in the future to meet the expected growth in world demand for n-3 oils.

Table 37.4 Typical analyses of algal oils. Adapted with permission from
Senanayake and Fichtali [25]

Parameter	DHASCO® oil	DHASCO®-S oil
Docosahexaenoic acid (g/kg)	min. 400	min. 350
Docosahexaenoic acid (%)	40–45	37–42
Arachidonic acid (g/kg)	–	–
Arachidonic acid (%)	–	–
Peroxide value (meq/kg)	0–0.5	max. 5.0
Free fatty acids (%)	0.03–0.1	max. 0.5
Moisture and volatiles (%)	0.0–0.02	max. 0.05
Unsaponifiable matter (%)	1–2	max. 4.5
Insoluble impurities (%)	below detection	below detection
Trans fatty acids (%)	below detection	below detection
Heavy metals (ppm)	below detection	below detection
Major fatty acids (%):		
10:0	0–0.5	–
12:0	2–5	0–0.5
14:0	10–15	9–15
16:0	10–14	24–28
16:1	1–3	0.2–0.5
18:0	0–2	0.5–0.7
18:1 n-9	10–30	0.5–3.0
18:2 n-6	0.4	0.5–1.3
20:0	<0.1	0.2–0.3
20:3 n-6	–	0–0.5
20:4 n-6	<0.1	0.5–0.8
22:0	0.1	0.1–0.2
22:5 n-6	–	12–16
22:6 n-3	40–45	37–42

DHASCO oil from *Crypthecodinium cohnii*; DHASCO-S oil from *Schizochytrium* spp.

37.5.1 Characteristics of microalgal oils

DHASCO® is an algal oil that is derived from the marine microalgal species *Crypthe-codinium cohnii*. The final oil contains approximately 40% (w/w) DHA. DHASCO® is a free-flowing liquid oil, which is yellow-orange in colour due to the co-extraction of carotene pigments. The final product contains about 95% TAG, with some diacylglycerols (DAG), and unsaponifiable matter, as is typical for all food-grade vegetable oils. Because of the controlled manufacturing process of algal oil, the potential for contamination with environmental pollutants or heavy metals is eliminated. The fatty acid composition of DHASCO® is reported in Table 37.4. The fatty acid profile of this algal oil is unique in that it contains no PUFA other than DHA, except a small quantity of linoleic acid (18:2 n-6 ∼0.5%) from high-oleic sunflower oil diluent [1]. DHASCO® has been used for supplementation of infant formulas. The unsaponifiable matter of DHASCO® is generally about 1.5% and is made up of mainly sterols [18]. The main sterol has been identified as the 4-methylsterol and dinosterol. The principal components of the sterol fraction in DHASCO® (e.g. dinosterol) are found in the normal metabolic pathway of cholesterol biosynthesis and have been identified in several common food sources including fish and shellfish. A study providing large amounts of the isolated unsaponifiable fraction of crude DHASCO® to rats concluded that these sterols had no adverse effects on growth or lipid metabolism [19]. The oil exhibits a remarkable oxidative stability. This is a result of the relatively low levels of pro-oxidant heavy metals

as compared to fis oil, and the favourable distribution of the DHA in the TAG molecules. Under typical storage conditions of –20 or 4°C, the DHASCO$^{®}$ is stable for several months. As a consequence, this oil has greatly accepted sensory qualities. When encapsulated in soft gelatin capsules of 250 to 500 mg each, the oil is stable for several years at room temperature before showing any change in the peroxide value (PV). Approximately 45% of the DHA found in algal oil are located at the *sn*-2 position of TAG molecules [20]. The TAG structure of algal oil is nearly identical to that of human milk, with respect to the positional distribution of DHA in TAG. Martin *et al.* [21] reported that in human milk about 50 to 60% of the DHA is preferentially esterifie at the *sn*-2 position of TAG. Thus, digestion and absorption of DHA in algal oil is expected to be similar to that of DHA in human milk fat.

DHASCO$^{®}$-S, also produced commercially, is a TAG oil extracted from the marine alga, *Schizochytrium* spp., which is enriched to about 40% (w/w) in DHA (Table 37.4). It is described as a yellow to light orange-coloured oil and contains greater than 90% (w/w) of TAG, with some DAG, free fatty acids, carotenoids, squalene, and phytosterols. Beta-carotene was identifie as the primary carotenoid component of the lipid fraction. The oil contains a range of fatty acids, including eicosapentaenoic acid (EPA, 20:5 n-3) and docosapentaenoic acid (DPA, 22:6 n-3), as well as DHA. However, DHA is the most abundant PUFA component of the oil. Compositional analyses of other components of the oils compare favourably with typical commercial edible oils. In general, the residual extraction solvent is undetectable, and there are no detectable *trans* fatty acids, pesticide residues, or heavy metals such as arsenic, mercury, and lead. The unsaponifiabl fraction of DHASCO$^{®}$-S is generally about 1.5% by weight and made up primarily of squalene, sterols, and carotenoids. These components are all present in the food supply. Cholesterol, brassicasterol, and stigmasterol were identifie as the major sterol components of the oil. The high DHA yields obtained with *Schizochytrium* spp. result in the production of a low-cost oil, which is used as a dietary supplement in foods and beverages, health foods, animal feed, and in aquaculture. Examples of foods and beverages containing DHA include yogurts, breakfast cereals, nutrition bars, hamburgers, sushi, breads, eggs, milk, soymilk, fruit juices, diabetic drinks, and nutrition drinks, etc. Other markets include prenatal supplements containing DHA for pregnant and nursing women, as well as dietary supplements for children and adults.

37.6   Conclusions

Among marine algae, which belong to the plant kingdom, one find some of the most value-added organisms in nature. Marine algae are an untapped resource with more than 30,000 species, of which fewer than 10 are in commercial production. Marine algae, which contain natural pigments such as carotenoids and phycobilins, have high protein and carbohydrate contents, and are rich in oil and fatty acids, including PUFA. Microalgae can be cultured in bioreactors to produce significan quantities of nutritionally important PUFA for extraction and purificatio at industrial scale.

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38 Preparative and industrial-scale isolation and purification of omega-3 polyunsaturated fatty acids from marine sources

Udaya Wanasundara

38.1   Introduction

Long-chain omega-3 (ω-3 or n-3) polyunsaturated fatty acids (PUFA) have become an important subject in both the scientifi community and our everyday lives, and we encounter them in pharmaceutical and/or health as well as food applications. The importance of n-3 PUFA for human health is apparent in several of their physiological effects. It is known that there is strong support for the use of n-3 PUFA in the secondary prevention of acute coronary syndromes. The beneficia effects of n-3 PUFA have been attributed to their ability to lower serum triacylglycerols (TAG), to increase membrane fluidit , and by conversion to eicosanoids to reduce thrombosis [1]. The n-3 PUFA are considered essential for normal growth and development throughout the lifetimes of humans and may play an important role in the prevention and treatment of hypertension, arthritis, other inflammator and autoimmune disorders, and various types of cancer [1,2].

A significan amount of docosahexaenoic acid (DHA; 22:6 n-3) (Fig. 38.1) is found in human milk. High levels of DHA are also found in human brain and retina. Therefore, DHA in breast-milk or in infant formula meets the requirements of developing human brain and visual parts [3]. The PUFA composition of cell membranes is largely dependent on their dietary intake. Therefore, consumption of appropriate amounts of n-3 PUFA needs to be considered. The n-3 PUFA, namely eicosapentaenoic acid (EPA; 20:5 n-3), docosapentaenoic acid (DPA; 22:5 n-3), and DHA (Fig. 38.1), may be acquired from marine sources or derived from α-linolenic acid (18:3 n-3) by a series of chain elongations and desaturation, albeit at only 1 to 5% . It has been suggested that the n-3 PUFA concentrates devoid of saturated fatty acids (SFA) are much better than marine oils themselves, since they allow keeping the daily intake of total lipids as low as possible [4].

Marine oils are abundant in n-3 PUFA and have traditionally been used as the raw material for preparation of n-3 PUFA concentrates. Because of the complex fatty acid composition of marine oils, n-3 PUFA in highly purifie form cannot be easily prepared by any single fractionation method. Usually a combination of methods is used, the combination of which depends on the fatty acid composition of the starting oil and the desired concentration and the purity of the n-3 PUFA in the end-product. This chapter covers the methods that may be used for isolation and purificatio of n-3 PUFA from source oils.
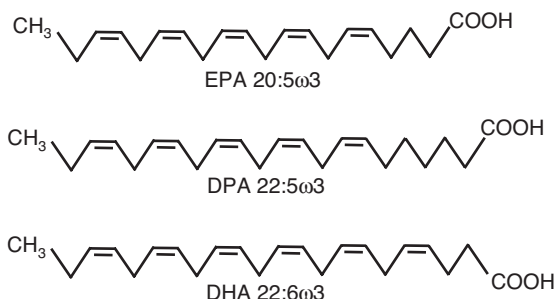
Fig. 38.1 Molecular structures of nutritionally important n-3 fatty acids (EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; and DHA, docohexaenoic acid).

38.2   Concentration methods of n-3 PUFA

Methods for concentration of n-3 PUFA are numerous, but only a few are suitable for large-scale production. The available methods include chromatography, fractional or molecular distillation, enzymatic splitting, low-temperature crystallization, supercritical flui extraction, and urea complexation (or urea adduct formation). Each method has its own advantages and drawbacks. This chapter provides a background to each of these methods. Some of the methods described in this chapter are now used on an industrial scale for the products with varying contents of n-3 PUFA [5]. The challenge is now to develop cost-effective procedures to produce n-3 PUFA concentrates to meet the growing demand.

The fatty acid separation is based on the specifi properties of each acid or acid group. The main properties, namely boiling and melting point differences and molecular configuration are utilized in developing separation techniques. The boiling point of a mixture of fatty acids varies significantl with the chain length of the fatty acids involved. This is used in fractional distillation as a means of separating short- and long-chain fatty acids. However, boiling point does not change much with the degree of unsaturation. In the other method, the melting point of fatty acids changes considerably with the degree of unsaturation and this could be used to separate a mixture of fatty acids into their saturated and unsaturated components. By changing the temperature of the mixture, fatty acids can be separated according to the degree of unsaturation at their respective crystallization temperatures. Molecular configuratio of fatty acids is also utilized in developing separation techniques, especially in urea complexation. While straight-chain saturated fatty acids with six or more carbon atoms are a readily form complex with urea, the presence of *cis* double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea.

38.2.1   Chromatographic methods

Although several gas chromatographic separation methods are available for analytical purposes, liquid chromatographic methods have been more applicable for preparative and industrial-scale separation of fatty acids or their esters. Simulated moving bed chromatography is a continuous purificatio technique based on a fl w of eluent moving counter-current to a constant fl w of solid-stationary phase and this technique can be applied for the purifi cation of EPA and DHA from source oil [6]. In order to obtain individual long-chain PUFA in concentrated form, chromatographic methods have been studied in detail. Nakahara *et al.*

[7] have reported the isolation of TAG containing DHA and DPA residues from marine microalgae (e.g. *Schizochytrium*) by reverse-phase high-performance liquid chromatography (HPLC) using acetone/acetonitrile as the mobile phase and octadecylsilane (ODS) as the stationary phase. Teshima *et al*. [8] used silver nitrate-impregnated silica gel column to separate EPA and DHA from squid liver oil fatty acid methyl esters with a purity of 85 to 96% EPA and 95 to 98% DHA and a yield of 39 and 48%, respectively. Use of HPLC columns comprising silica gel and silver or magnesium ion has been reported by Corley *et al*. [9] to isolate TAG rich in DHA from marine algal oils. Adlof and Emiken [10] were able to enrich the n-3 content of commercial n-3 PUFA concentrates from 76.5 to 99.8% using isocratic elution from a silver resin column. In another study, the same authors fractionated 100 mg of concentrated fis oil methyl esters containing 29.1% EPA and 20.5% DHA into fractions of 87.7% EPA and 95.4% DHA, with increasing amounts of acetonitrile (0–30%, v/v) in methanol. They also fractionated non-enriched menhaden oil methyl esters and fatty acids that contained approximately 12.5% EPA and 11.1% DHA. The separation was done isocratically using 40% (v/v) acetonitrile in acetone to yield one eluted fraction containing approximately 69% EPA and DHA in total. Several patents have been granted for different types of adsorbents and techniques to separate fatty acids. Methyl esters are preferred to fatty acids as they yield higher purity finishe products and require milder conditions during synthesis.

38.2.1.1 *Counter-current chromatography*

Counter-current chromatography (CCC) and/or centrifugal partition chromatography (CPC) has been used for the isolation of PUFA. CCC or CPC is a liquid chromatographic technique that utilizes liquid-liquid partition, counter-current distribution of a solute mixture between two liquid phases, in the absence of a solid support, to perform separation of a complex mixture of chemical substances [11]. Since CPC does not use a solid support as the stationary phase, the possibility of irreversible retention of highly retentive sample components is eliminated. For this reason, this chromatography assures almost 100% recovery of the eluted compounds. Any two-phase solvent system may be used; many partition systems can be employed with non-toxic, commonly available solvents. Decomposition and/or denaturation of valuable components such as PUFA, often encountered with conventional packed-bed chromatographic columns, are virtually non-existent under the mild operating conditions used in CPC. The other advantage of CPC is the low solvent consumption and that both normal and reversed-phase elution may be conducted with the same solvent pair in a closed system. CPC may be readily adapted for large-scale continuous separations. In addition, environmental problems are minimal and solvent may be completely recovered and recycled. Details of the CPC instrument and its function and also solvent selection have been described by Wanasundara and Fedec [11].

A CPC method has been developed to purify DHA from algal oils [11]. The algal oil used contained 39.7% DHA and 15.2% DPA (n-6) with several other fatty acids. The free fatty acids (FFA) of the algal oil were eluted with hexane/methanol/water (100:95:5, v/v/v) two-phase solvent system in normal phase ascending mode. Under these conditions, it was possible to purify DHA up to 84.6% and DPA up to 84.9% (Fig. 38.2). However, under these conditions 14:0 fatty acid co-eluted with DHA, therefore a higher DHA purity could not be achieved. In order to isolate ultra pure DHA (fin chemical grade), pre-purificatio of algal oil-FFA was carried out by urea complexation (Section 38.2.7) in order to remove the co-eluting 14:0 fatty acid. When the pre-purifie FFA was used in CPC, it resulted in ultra-pure DHA (99%) with a high degree of recovery (Fig. 38.2).
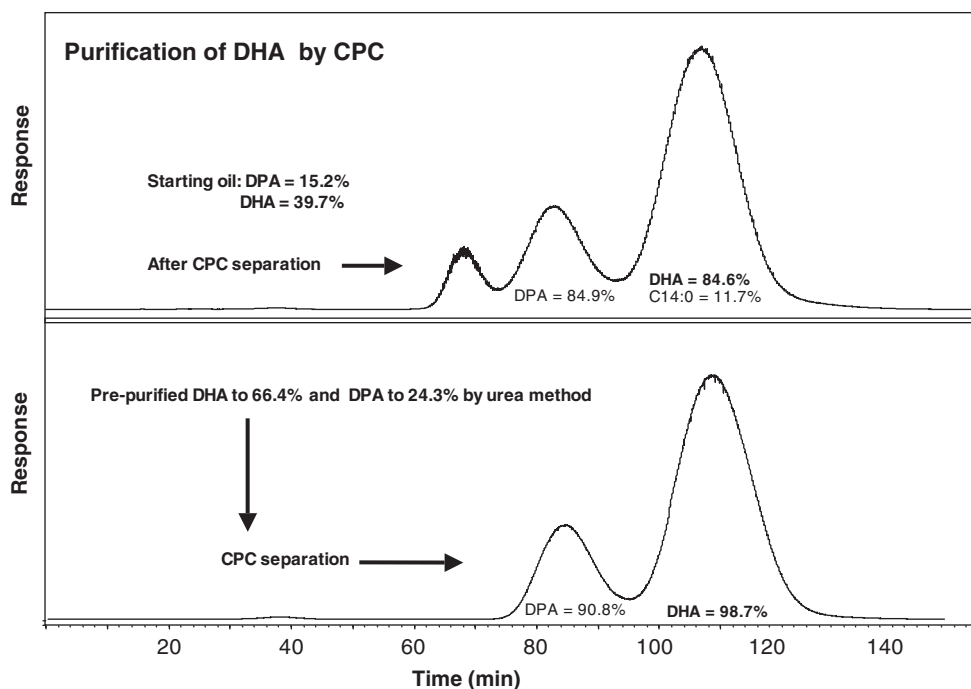
Fig. 38.2 Purification of n-3 fatty acids by centrifugal partition chromatography (CPC) (DHA, docohexaenoic acid and DPA, docosapentaenoic acid).

Bousquet *et al.* [12] have tested CPC separation of EPA and DHA from a microalgal oil and were able to isolate pure EPA and DHA from this oil with excellent yields. The firs separation used heptane as the stationary phase and acetonitrile/water (3%) as the mobile phase; this system was able to remove fatty acids of minor importance, leaving a mixture of four major PUFA. From a crude acid mixture (2.4 g), a mixture (1 g) of the following four fatty acids (e.g. 18:3 (43%), 18:4 (7.5%), EPA (45%), and DHA (4.5%)) were obtained. This mixture was subjected to another separation using heptane as a stationary phase and methanol-water as the mobile phase (bi-phasic solvent system of heptane/methanol/water; 500:415:85, v/v/v). Under these conditions, a good separation was achieved and isolation of pure EPA and DHA resulted in an excellent yield [13]. The CPC may also be applied to separate EPA and DHA ethyl esters with a solvent system comprised of hexane/dichloromethane/acetonitrile (5:1:4, v/v/v).

CPC, a powerful process-scale separation technology for separation, isolation, and purifi cation of n-3 PUFA, is also complementary to HPLC. Although capital investment for a CPC often runs higher than that for HPLC, operating costs are generally an order of magnitude lower than when the same separation is performed with conventional liquid chromatography or preparative HPLC. Another advantage is that the separation may be carried out under a blanket of nitrogen or another inert gas, in order to protect substances prone to oxidation. CPC is indeed a powerful tool, which is emerging in several areas of lipid chemistry. So far, it has demonstrated its potential for the separation of lipid molecules at the level of a few grams and is expected to develop into production at kilogram levels [11].

38.2.2 **Supercritical fluid extraction method**

Supercritical flui extraction (SFE) is a relatively new separation process that may circumvent some of the problems associated with the use of conventional separation techniques. The most commonly used solvent is carbon dioxide, because it has moderate critical temperature and pressure (31.1°C, 73.8 bar) and is inert, inexpensive, non-flammable environmentally acceptable, readily available, and safe. The separation of PUFA by SFE is dependent on the molecular size of the components involved rather than their degree of unsaturation. Therefore, a prior concentration step is needed to achieve a high concentration of PUFA in the fina product [14]. Oils to be used for n-3 concentration by SFE require preparation steps of extraction, hydrolysis, and esterificatio by conventional methods [15]. The use of supercritical fluid for extraction of oil and concentration of n-3 PUFA from fis oil and seaweed has been reported [16]. Some enhancement of the content of n-3 PUFA present in fis oil TAG has been reported. Stout and Spinelli [17] have demonstrated that fis oil esters could be fractionated by SFE to produce oil with a DHA content of 60 to 65%. The fractionation of FFA using SFE has also been reported [18]. Use of extremely high pressures and high capital costs might limit the widespread use of this method for concentrate production to the larger processing companies. A detailed review of SFE and fractionation of fis oils has been given by Nilsson [19].

38.2.3 **Low-temperature crystallization method**

It is generally understood that the solubility of any given acid is closely related to its melting point and to a certain degree dependent on the nature of the solvent. Fractionation by crystallization, using the melting point differences of fatty acids or TAG is done in two ways. Dry fractionation, also known as low temperature crystallization, is the enrichment of oil with more unsaturated TAG by removing saturated and high melting components at low temperatures. The other method is solvent fractionation (crystalizaton), which involves use of organic solvents such as acetone or hexane in order to improve the yield of each fraction.

Crystallization is a mild procedure and especially suitable for PUFA, but the separation of PUFA from one another works less satisfactorily than the separation of unsaturates from saturates [20]. Isolation of PUFA by crystallization is best performed using organic solvents at very low temperatures, making the process less attractive for industrial application. Crystallization of SFA at low temperatures has been in practice since 1940s and has developed into a commercial process for separation of SFA from natural TAG sources. Solvent crystallization of fatty acids is an indispensible method for preparing pure fatty acids. This method requires the least number of unit operations and the simplest equipment [21]. Briefl , the process consists of cooling the oil or fatty acids in a solvent, holding for a specifie period of time, and removing the crystallized fraction by filtration

The low temperature crystallization process may be carried out on the neat liquid in the absence of a solvent or in a selected solvent/solvent mixture. Crystallization of SFA from a solution of fatty acid mixture in organic solvents, especially a polar type, is a promising way of separation. It has been reported that use of different organic solvents and temperatures affects the concentration of PUFA [22]. With proper choice of solvent and temperature, PUFA can be concentrated into a non-crystallized fraction. Studies carried out on solvent crystallization of PUFA from seal blubber oil (SBO) showed that fatty acids in the free or TAG forms can be concentrated into the non-crystalline fraction [23,24]. Table 38.1 shows the enrichment of total n-3 PUFA following low temperature crystallization of SBO in the TAG

Table 38.1   Fractionation of n-3 PUFA (%) from seal blubber oil (SBO) by low temperature crystallization in different solvents [24,46]

Fractionation temperature (°C)	TAG form		FFA form	
	Hexane	**Acetone**	**Hexane**	**Acetone**
	Total n-3 PUFA content of original SBO is 20.1%			
−10	23.2	23.5	23.8	24.3
−20	23.9	26.9	24.5	25.4
−40	26.2	36.4	31.0	40.6
−60	30.5	43.8	58.3	56.8
−70	35.1	47.9	66.7	46.8

Abbreviations: TAG, triacylglycerols and FFA, free fatty acids.

form using hexane and acetone as solvents. The content of n-3 PUFA in the non-crystalline fraction (the concentrate) was increased with lowering of the crystallization temperature. Under all temperature conditions, acetone afforded the highest concentration of total n-3 PUFA. Low temperature crystallization of SBO, in the FFA form, at –60 and –70°C in hexane, resulted in total n-3 PUFA contents of up to 58.3 and 66.7% in the preparation with concentrate recoveries of 39.0 and 24.8%, respectively. However, the content of total n-3 PUFA in acetone increased up to 56.8 and 46.8%, but the recovery of the concentrates was 15.9 and 12.9%, respectively [24].

38.2.4   Fatty acid–salt solubility method

In 1828, Gusserow introduced a method in which lead salts or soaps of fatty acids in diethyl ether were separated, depending on their solubility differences. Saturated and unsaturated fatty acids formed salts with metal ions (e.g. Li, Na, and K) whose solubilities in water and organic solvents varied with the nature of the metal ion and the chain length and the degree of unsaturation. The alkali salts of SFA crystallize more readily than those of PUFA containing four or more double bonds when the saponifie solution is cooled. Therefore, concentration of n-3 fatty acids from PUFA-rich oils in the form of fatty acid salts may be achieved by employing a lower alcohol (e.g. ethanol) using solubility differences of the salts. In order to obtain a high content of total n-3 fatty acids with a good recovery, the water content of the medium for this procedure should be maintained at a 3% level. Han *et al.* [25] were able to increase the total n-3 fatty acids of sardine oil from 33.2 to 75.9% using the salt solubility differences of fatty acids in ethanol. Studies carried out in our laboratory, by applying the salt solubility method on seal blubber oil, demonstrated that both EPA and DHA can be increased when SBO convert to sodium salt in the 99% ethanol medium (Unpublished data) (Table 38.2).

Table 38.2   Enrichment of PUFA in seal blubber oil (SBO) by fatty acid-salt solubility method

Fatty acid (%)	Original SBO	Ethanol (99%)	Ethanol (90%)
EPA	6.41	11.3	6.73
DHA	7.58	19.0	10.0
Total n-3 PUFA	20.1	40.8	24.2

Abbreviations: EPA, eicosapentaenoic acid; DHA, docohexaenoic acid.

38.2.5 Distillation method

Separation of fatty acid by distillation depends on the relative volatility of individual fatty acids in a mixture. This method takes advantage of differences in the boiling point and molecular weight of fatty acids under reduced pressure. Distillation of SFA and fatty acid esters is a common fractionation technique. However, the heat liability of PUFA prevents the use of distillation through traditional columns. Short-path distillation or molecular distillation uses lower temperatures and short heating intervals. The most widely used distillation procedure is fractional distillation of methyl or ethyl esters under reduced pressure (0.1–1.0 mmHg). Even under these conditions, moderately high temperatures are required; the more highly unsaturated acids, especially n-3 PUFA, are more prone to oxidation, polymerization, and isomerization of double bonds. Heated columns packed with glass helices or some form of metal packing are in common use, despite the disadvantage of a significan hold-up and pressure drop through the column. Spinning band columns do not suffer from these disadvantages. Distillation at still lower pressures has been used in the isolation of some highly unsaturated acids.

Stout *et al*. [26] emphasized the practical difficult of concentrating only n-3 PUFA from fis oil in the natural TAG form. The distillation of menhaden oil, as such, concentrated only EPA from an initial of 16.0 to 19.5%. However, distillation of its ethyl esters increased the EPA content from 15.9 to 28.4%. The concentration of DHA was even more dramatic. While DHA doubled from 8.4 to 17.3% in the TAG form, in the simple alkyl esters form it increased from 9.0 to 43.9%.

Exposure of long-chain n-3 PUFA to high temperatures during distillation may induce hydrolysis, thermal oxidation, polymerization, and isomerization. Possible degradation products of long-chain PUFA are cyclic fatty acids and high-molecular-weight polymers [27]. Privett and Nickell [28] found marked decomposition of arachidonic acid (20:4 n-6) when it was distilled slowly in a spinning band column. Therefore, design of a method for preparation of n-3 PUFA concentrates, which involves low process temperature and time to minimize thermal damage, is desirable.

38.2.6 Enzymatic methods

Application of enzymes, especially microbial lipases to concentrate n-3 PUFA has been a focus of attention in both academic and industrial circles. Lipases may catalyze esterification hydrolysis, or exchange of fatty acids in esters [29]. These processes can be selected by choosing appropriate substrates and reaction conditions. Since enzymatic reactions occur under mild temperature and pH conditions and under ambient pressure, they generally require less energy and are conducted in equipment of lower capital cost than many other chemical processes. Another advantage of enzymatic process is related to the selectivity of many lipases, which allows obtaining products that are difficul to produce by more conventional chemical reactions.

38.2.6.1 *Lipase-catalyzed hydrolysis*

Much attention has been paid to enriching the n-3 PUFA content in the acylglycerols using microbial lipases. The reason is that certain microbial lipases have lower activity towards long-chain PUFA such as EPA and DHA. Such lower reactivity can explain the mechanism of lipase-catalyzed reactions for enriching the n-3 PUFA content of marine

oils. The presence of *cis* carbon–carbon double-bonds in the fatty acids results in bending of the chains (Fig. 38.1). Therefore, the terminal methyl group of the fatty acids lies close to the ester bond, which may cause a steric hindrance effect on lipases. The high bending effect of EPA and DHA, due to the presence of 5 and 6 double-bonds, respectively, enhances the steric hinderance effect, therefore lipases cannot reach the ester-linkage between these fatty acids and the glycerol moiety. However, saturated and monounsaturated fatty acids (MUFA) do not present any barriers to lipases and thus may be easily hydrolyzed. Therefore, selectivity of a lipase for EPA and DHA allows their separation and concentration from other components present in the remaining portion of marine oils.

Microbial lipases from *Aspergillus niger (AN), Candida cylindracea (CC), Pseudonas* spp. *(PS), Chromobacterium viscosum (CV), Rhizopus delemer (RD),* and *Rhizopus javanicus (RJ)* have been widely used in modifying PUFA-rich oils [30]. The fatty acid specificit of lipases (discrimination of PUFA over short-chain fatty acids) is a crucial factor when considering the application of enzymes to modify marine oils rich in PUFA [31]. A Japanese patent [32] describes a method based on the discrimination of lipases on EPA and DHA for preparation of n-3 PUFA concentrates. Ethyl esters from selected fis oils, such as those of sardine and mackerel, were hydrolyzed with various lipases [*CC, AN*, and *Mucor miehei (MM)*]. Selective hydrolysis afforded ethyl ester concentrates of up to 25% EPA and 17% DHA after separation of the hydrolyzed fatty acids. SBO, menhaden oil (MHO) [33,34], and tuna oil [30] have shown that lipase-assisted hydrolysis may enrich n-3 PUFA in the acylglycerols of the unhydrolyzed fraction. In MHO, the total content of n-3 PUFA was increased from 30% in the original oil to 45.7, 45.8, and 42.2% after 75 hours hydrolysis by *RO-*, *CC-* and *GC*-lipases, respectively. In SBO, maximum increase in the content of total n-3 PUFA, from 20.2 to 45.0%, was achieved using *CC*-lipase under similar experimental conditions [23,35]. Although selective hydrolysis was found to be a simple method for concentrating n-3 PUFA in marine oils, the efficien y of enrichment was usually not high. The highest content obtained was less than 50%. Therefore, newer methods using lipase-catalyzed reaction or combination of enzymatic method with other fractionation methods are required.

38.2.6.2 *Lipase-catalyzed esterification*

The TAG form of PUFA is considered to be nutritionally more favourable than methyl or ethyl esters of fatty acids, because experimental results have shown impaired intestinal absorption of methyl or ethyl esters of n-3 PUFA in laboratory animals. Yang *et al*. [36] have shown that methyl and ethyl esters are hydrolyzed slower than their corresponding TAG. From a marketing point of view, TAG of PUFA are often promoted as being more "natural" than other fatty acid derivatives. In order to include preferred fatty acids, the TAG lipase-catalyzed esterificatio of fatty acids is widely employed and structured lipids with high biological value are produced.

Several researchers have reported that lipase-catalyzed esterificatio affords a high degree of incorporation of targeted fatty acids into the glycerol molecule. Osada *et al*. [37] have employed *CV*- and *CC*-lipases for direct esterificatio of glycerol with individual FFA, including EPA and DHA. The *CV*-lipase exhibited superiority over that of *CC*-lipase as it afforded a reaction yield of 89 to 95%. With the latter lipase a reaction yield of 71 to 75% was obtained for all fatty acids examined except for DHA which gave a 63% esterificatio level. Glycerolysis of n-3 PUFA, obtained from SBO, was studied using *CV*-lipase [38]. The degree of synthesis reached was up to 94% and the amounts of mono-, di-, and triacylglycerols in the

product were 13.8, 43.1, and 37.4%, respectively. All these studies point to the fact that water content in the reaction medium is a crucial factor determining the extent of the esterificatio reaction. High water content in the reaction medium shifts the chemical equilibrium towards hydrolysis, whereas reduced water content shifts the equilibrium towards esterification The optimum content of water in the esterificatio reaction should be kept to a minimum in order to discourage occurrence of partial hydrolysis of products and formation of glycerol as well as mono- and di-acylglycerols. However, the content of water in the reaction medium should be sufficientl high in order to prevent enzyme deactivation. The water requirement for different enzymes varies considerably, typically from 1 to 4% for inter-esterificatio reactions [39]. Starting with previously enriched material (high content of targeted fatty acids by other means such as urea fractionation), it is possible to obtain very high levels of incorporation [40].

Preparation enzyme-assisted n-3 PUFA concentrates from marine and algal oils has attracted increasing attention. A number of companies claim to be using the lipase-catalyzed reaction for the large-scale commercial production of the concentrates. All of the enzymatic reactions required for the production of n-3 PUFA concentrates can be conducted under ambient temperatures, normal pressure, and nitrogen-protected environment, regardless of whether they involve hydrolysis or esterification Therefore, use of enzymatic processes for production of n-3 PUFA concentrates is safer and more efficien that other methods described in this chapter.

38.2.7 Urea complexation method

Urea has the unique property that forms solid complexes (adduct) with straight-chain hydrocarbons. Urea alone crystallizes in a tightly packed tetragonal structure with channels of 5.67 Å diameter. However, in the presence of long straight-chain molecules, it crystallizes in a hexagonal structure with channels of 8 to 12 Å diameter within the hexagonal crystals [41]. The channels formed, in the presence of long-chain unbranched molecules, are sufficientl large to accommodate aliphatic chains. While straight-chain SFA with six carbon atoms or more are readily adducted, the presence of double bonds in the carbon chain increases the bulk of the molecule and reduces the likelihood of its complexation with urea [24,42]. Therefore, formation of urea inclusion compounds depends on the degree of unsaturation of the fatty acids. During this process, the n-3 PUFA containing oil (aclyglycerol) is split into fatty acids using alcoholic KOH or NaOH. The FFA, which are mixed with an alcoholic (methanol or ethanol) solution of urea, are then allowed to cool to a particular temperature, depending on the degree of concentration desired. The SFA, monoenes, and to a lesser extent, dienes are crystallized with urea and non-crystallized fatty acids in the solution can be separated by filtration The liquid or non-urea complexing fraction (NUCF) is enriched with n-3 PUFA. Alternatively, this procedure can be carried out using methyl or ethyl esters of fatty acids rather than FFA. There are advantages and drawbacks to each of these options. For example, fatty acids are more soluble in alcohol than their corresponding esters, hence they require a much smaller volume of alcohol for processing. If the ester form of fatty acids is chosen, the re-esterificatio step of the concentrates is eliminated.

It has been reported that complete removal of SFA by urea complexation is impossible since some of the shorter chain SFA do not complex with urea during the crystallization process [23,43]. Long-chain MUFA, especially those of the C20 and C22, form complexes with urea more readily than those of the shorter chain SFA (C10 and C12), thus the amount of MUFA in UCF may be increased depending on the reaction conditions. Complex formation

is exothermic, but requires dissolution of both urea and fatty acids. Methanol and ethanol are preferred solvents for small-scale fractionation, but Han *et al*. [44] found that water may offer the best choice of solvent for large-scale operations.

Urea complexation processing of SBO was carried out in order to concentrate its n-3 PUFA [23,42]. Among the major n-3 PUFA, DHA was found almost exclusively in the NUCF under selected experimental conditions. Although most of the EPA was recovered in the NUCF, a small proportion of it was invariably complexed with urea and ended up in the UCF. Urea complexation of SBO, under optimum condition, gave a total PUFA content of 92.3% in the NUCF. However, it was difficul to remove all of the SFA in order to obtain a 100% PUFA concentrate. Ratnayake *et al*. [43] have also reported that complete removal of SFA by urea complexation may be impossible, since some of the shorter chain SFA do not complex with urea during the crystallization process. The enrichment of total n-3 PUFA in the concentrate, and its overall recovery, varied inversely with increasing urea-to-fatty acid ratio as well as crystallization at lower temperatures. Therefore, these experimental variables should be carefully controlled in order to achieve a maximum content of total n-3 PUFA in the concentrate with a reasonable recovery [45,46]. Use of urea complexation in combination with chromatographic separation allows preparation of fatty acids with a high degree of purity (Fig. 38.2). The urea complexation method of fatty acid concentration may be applied to industrial-scale operations with minimum capital cost. The Norwegian company Pronova Biocare AS uses this method for fractionation of ethyl esters [5]. Omega-3 PUFA concentrates produced by the urea method do not have a purity level suitable for direct use in supplements as well as in pharmaceuticals. Therefore, such concentrates will be further purifie using molecular distillation [5].

38.3   Conclusions

Production of n-3 PUFA concentrates from marine sources may be achieved using a number of methods described in this chapter. Since the prepared concentrates are highly prone to oxidative deterioration, preventive measures should be considered to address this issue. The protection has to begin with the starting oil, and continue all the way through the production process. The stability of prepared concentrates can be improved by addition of suitable antioxidant(s), as well as other physical means such as microencapsulation and gel capsulation.

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39 Marine oil processing and application in food products

Fereidoon Shahidi

39.1 Introduction

Polyunsaturated fatty acids (PUFA), particularly those of the omega-3 (n-3 or ω-3) family, provide important health benefit to consumers but also present the scientists and technologists with a difficul challenge in delivering the highly unsaturated fatty acids (HUFA) foods that are appealing and do not have off-fl vours associated with their oxidation products [1]. Interest in n-3 fatty acids as health promoting dietary components/supplements has expanded dramatically in the last three decades or so [2–4]. There is a rapidly growing body of literature illustrating cardiovascular [5–7] and a myriad of other health benefit of HUFA, with respect to inflammator diseases [8], certain type of cancer as well as type II diabetes [9], and mental health [10–12]. Evolutionary assessments suggest that most Western populations are consuming far less n-3 fatty acids than historically and much less than appears to be nutritionally desirable [13,14]. The best sources of n-3 fatty acids are the body of fatty fish liver of white lean fish blubber of marine mammals, as well as micro- and macroalgae. However, high oil fis are the best sources of n-3 fatty acids, but the consumption of fis is often too low to meet the requirements. Efforts to supplement foods with n-3 fatty acids have been slow because of off-fl vours associated with the oils, especially during storage. The n-3 rich fis oils are extremely labile to oxidation, thus requiring control of oxidation and off-fl vour development [15,16,25]. Fortunately, recent advances in the microencapsulation and coacervation technologies have allowed production of stabilized products that could be introduced into foods without being degraded, as the microcapsules that include the oils are fairly stable and some are able to release their content only after reaching the gastrointestinal tract [33]. This chapter provides a cursory account of the processing aspect of marine fis oils and their application into food products.

39.2 Marine oil processing

Crude fis oil is often a by-product of the fis meal industry [17]. The type of fis caught for the meal industry are white anchovy, black anchovy, sardine, mackerel (Chile and Peru), capelin, blue whiting, herring, menhaden, sandeel (Iceland and Norway), and sprat (Denmark).

Typical commercial products receiving this process include shark liver oil, salmon oil, cod liver oil, tuna oil, menhaden oil, and anchovy oil, etc.

The extraction of the oil from the fis is done through a grinding process. Whole fis or processing discards such as heads and tails are being used for fis oil and meal production [18]. The crude material is passed through a rotating grinder where the oil is freed and pressed out. At this stage, the crude oil contains dispersed matters, water, and impurities, as well as oxidation products such as aldehydes and ketones that must be removed by further processing before the oil is suitable for consumption. The crude oil can be used for the tanning industry, margarine oil (hydrogenated), supplement industry, and for aquaculture. Typically, the crude oil is stored up to three years in drums with nitrogen flushin and then sold to a refine [4].

Some of the highest levels of eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) fis oil come from the anchovy and the sardine, which contain 21% EPA and 9% DHA for the anchovy and 16% EPA and 16% DHA for the sardine. These fis are caught in waters off the coast of Peru and Chile.

The firs process used in the refinin of crude fis oil is an alkali-refinin step (Fig. 39.1). Food grade dilute sodium hydroxide (baume) is added to the crude oil to convert the free
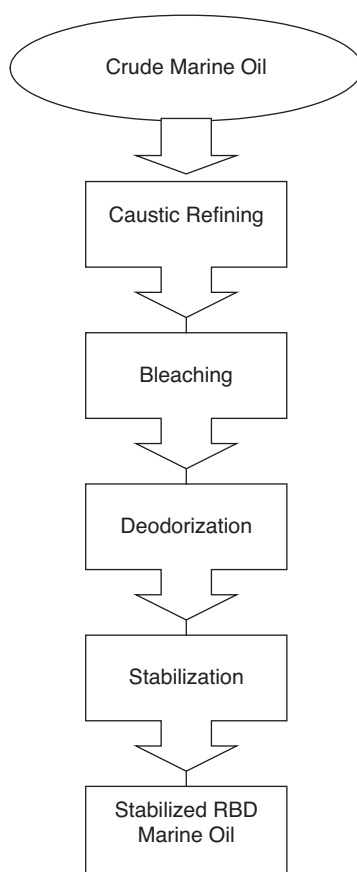
Fig. 39.1 Production of refined, bleached, and deodorized (RBD) and stabilization with antioxidants of marine oils.

fatty acids (FFA) to soap. The FFA soaps then precipitate (soap stock) and are removed by centrifugation. The remaining soap may be removed either by water washing or Trisyl-silica treatment. The oil is then filtere through fi e-micron filters which remove any remaining proteins and other particulates.

Bleaching is a simple process where the oil is mixed in a tank with bleaching clay and the mixture is heated to 50 to 60°C under vacuum (Fig. 39.1). If the refine oil is too dark to bleach, activated carbon may be added to the bleaching in addition to clay. The clay adsorbs the colour components of the oil leaving clear oil. In this process, the hydroperoxides left in the oil are decomposed to carbonyl compounds by the bleaching clay.

The deodorization process used for most marine oils is molecular distillation. The molecular distillation process removes volatile contaminants such as pesticides, remaining FFA, water, aldehydes, and ketones as well as some of the desirable components such as tocoperhols, etc. The deodorization process (Fig. 39.1) generally is a two-stage process.

1) The firs stage in the molecular distillation is conducted in a wiped fil still, which consists of two concentric cylinders. The inside cylinder contains oil and wipers, which are rotating up to 200 rpm. The fast rotating wipers disperse the oil into a thin layer against the wall of the cylinder. The top of the cylinder is connected to a vacuum. The vacuum and mild heat removes the volatile components from the thin fil of oil on the wall. The vacuum also keeps the oxygen levels low so the oxidative damage is minimized despite the heat applied in the process. At this stage, the oil and heavy contaminants are collected at the bottom.

2) In the second stage of deodorization the oil is heated in the outer portion of the still and a condenser in the middle of the "inside cylinder" is chilled to trap volatiles. The outside cylinder is heated up to 250°C, which strips more volatiles from the oil and they will be condensed on the chilled inner portion. The vapours are condensed on the condenser and drop to the bottom of the cylinder for removal through the central drain of the condenser. The liquid oil moves down the wall of the inside cylinder and exits where it is cooled through a heat exchanger.

However, certain suppliers may make use of silica gel or similar products in order to also adsorb the unwanted contaminant from the oil prior to deodorization, in place of or in addition to the refinin and bleaching. The deodorization process is also carried out at a lower temperature compared to those of vegetable oils. Finally, the refined bleached, and deodorized (RBD) oils may be subjected to stabilization (Fig. 39.1) by the addition of appropriate antioxidant(s)/chelator(s) and/or other potential stabilizer(s).

39.3 Enriched omega-3 oils

For some applications, particularly supplements, enrichment of the EPA and DHA in marine and algal oils is desirable [19]. The enrichment process also reduces the levels of myristic (14:0) and palmitic (16:0) acids that might be present in fis or algal oil. These fatty acids are known to be hypercholesterolemic. Thus, reduction of their levels greatly enhances the desirability of the fina product. EPA/DHA concentrates are prepared starting with the high-quality oils described above. There are different methodologies for production of n-3 concentrates [19]. A comprehensive review of n-3 concentration is provided elsewhere in this book (Chapter 38). These may include urea complexation, lowering of temperature,

Table 39.1 Omega-3 fatty acids in disease risk reduction

Disease/conditions	Example
Cardiovascular	Arrhythmias, lowering of triacylglycerols (TAG), etc.
Mental health	Depression, schizophrenia, Alzheimer, mood stabilizer, bipolar, and attention deficit/hyperacting disorder (ADHD)
Inflammatory	Bowel disease, Crohn's disease, ulcerative colitis, organ transplant, arthritis, and asthma
Cancers	Colon, breast, prostate, renal, etc.
Eye	Macular degeneration, etc.
Skin	Psoriasis, eczema, etc.
Diabetes	Type 2 diabetes

supercritical extraction, centrifugal partition chromatography, and distillation, etc.; most of which are generally carried out on the fatty acids after the hydrolysis of the oils [20]. In addition, enzymatic processes also yield concentrates, although often directly on the triacylglycerols (TAG). The resultant oils may be in the FFA, alkyl ester, or acylglycerol forms, but ethyl esters and acylglycerol forms are often marketed. Generally, the firs step is to convert all of the fatty acids in the fis oil to esters, with ethyl esters being the preferred form. The process of inter-esterificatio to ethyl esters consists of adding ethanol and sodium hydroxide to the oil heated to 80°C.

Distillation or urea complexation is then used to separate the fatty acid esters. Generally, the distillation process is usually carried out twice. The firs distillation yields EPA/DHA concentrations of around 25% EPA and 18% DHA. The second distillation further concentrates the esters to 30% EPA and 20% DHA. However, there are a number of processes where products with desired ratios of EPA and DHA are prepared, depending on their intended use.

The ethyl esters so produced may be converted to acylglycerols with or without the addition of other fatty acid sources. Thus, the re-esterificatio process is accomplished by adding glycerol or ethanol (to create a blend), and sodium methoxide as the catalyst. After this reaction, most of the molecule is back to a TAG form; in addition, mono- and diacylglycerols (MAG and DAG) may be present.

The concentrates so produced are generally used for supplements and for intervention studies. For example, EPA concentrate is used for addressing schizophrenia and certain other mental disorders while concentrates containing different ratios of EPA and DHA are used for selected applications (Table 39.1).

39.4 Application of the omega-3 fatty acids/oils

A comprehensive review of the current state of knowledge on the chemistry of HUFA is provided elsewhere in this book (Chapters 36–38). The health benefit and alternatives to processing and stabilization of the highly unsaturated oils have also been discussed [21,22]. Clearly, there is an opportunity in the future to fin new creative ways to stabilize these highly beneficia oils for delivery in food systems, above those already available to the industry. To gain consumer acceptance, the foods supplemented with marine oils must taste as good as or better than current products and be delivered to the consumer at a modest cost. Supplements offer an alternative approach, but cost and compliance are continuing issues with their use in some cases.

Table 39.2 Selected food products enriched with omega-3 oils

Category	Food products
Baked goods and mixes	Breads, cereals, pastas, crackers, noodles, etc.
Dairy products (fresh/frozen) and cheeses	Milk, yogurt, yogurt drinks, butter, cheese, etc.
Confectionary	Sweets, candies, cakes, bars, etc.
Spreads and dressings	Margarine, spreads, salad dressings, mayonnaise, etc.
Meat, poultry, and seafoods	Fabricated products
Fruit juices and other non-alcoholic beverages	Orange juice, fruit juice, etc.
Infant formula	Milk, baby formula, etc.
Fats and oils	Enriched fats and oils products
Soups, gravies, and sauces	Fresh or dried soup mixes, sauces, etc.
Egg-based products	Powdered and fresh products, etc.

An important area of the use of the n-3 oils is infant formula [23]. With respect to regulatory issues, in May 2001, the US Food and Drug Administration (FDA) issued a generally recognized as safe (GRAS) notificatio regarding the use of a DHA oil from algal sources, in infant formula. The addition of n-3 to infant formulas has resulted in products that are available around the world. Another company markets its Omega product line in South America and the Far East. Meanwhile, several other companies have or are in the process of having their n-3 fatty acids/oils used in infant formulas and other products.

The use of n-3 oils in foods has generally been for those foods that are used within a relatively short period from the time of their manufacture. These commercial products include beverages (different levels), cheese (130 mg EPA+DHA per 100 g, bread (29 mg long-chain n-3 PUFA per 100 g), and eggs (>200 mg per 100 g). Mayonnaise, sweet fl voured nutrition bars, cream cheese, and drinkable, as well as cup yogurt are also marketed in n-3 enriched forms. Recently it was announced that a major cereal signed a 15-year supply agreement with an n-3 producer and is working on adding n-3 to its products. In general, all food items can be enriched (Table 39.2).

As mentioned earlier, fl vour remains the major obstacle in certain cases that continues to affect progress on the enrichment and fortificatio of food products with n-3 oils. For example, low pH combined with high iron content (caused by egg yolks) accelerated oxidation and reduced the shelf-life of the n-3 fatty acids in mayonnaise. Presence of copper and peroxides in foods can also be a problem in accelerating the oxidation of n-3 fatty acids. From extracting oils out of a fresh fis to the intake in the daily household of EPA +DHA, considerable handlings have to be done and these should not affect oxidation at any stage that could generate off-odour or taste in the products. Fortunately, there are solutions to these problems. Oils high in n-3 fatty acids may be spray-dried and oil encapsulated in a dry matrix with very low exposure to surface oxidation. Oxidation of n-3 fatty acids can also be controlled by pH adjustment in emulsions or by producing low-viscosity emulsions for ease of handling and incorporation into water-based foods.

For food enrichment, functional marine oil can be incorporated in all kinds of foods as pure marine oil or as a mixture with vegetable oils. In practice, the natural antioxidants in the vegetable oil protect the fis oil against oxidation.

Addition of mixed tocopherols (antioxidants) and ethylenediaminetetracetic acid (EDTA, a metal chelator) has proven effective in improving oxidative stability of n-3 fatty acids. As mentioned earlier, most companies market fis oil-based products, often packaged with proprietary microencapsulation technologies. Marine oils can also be incorporated as powder

through maltodextrin powder (50%) or egg powder (45%). Other marine oils for dietary supplements include cod liver oil, salmon oil, and tuna oil.

An important growing area for the use of n-3 oils is the beverage sector. Globally, the market for n-3 enriched drinks is growing steadily, especially in North America, according to a recent report, which claims the n-3 enriched beverage market generated global sales in the region of over US$7 billion during 2006. North America accounted for nearly 33% of global volume in 2006, followed by Western Europe at 24% and Asia/Australasia at 23%. While North America's consumers generally enjoy lower average milk and juice/juice-based products prices than their West European counterparts, other types of n-3 enriched soft drinks are sold at a premium. A California-based company formulates a range of milk-based foods and beverages, including orange juice and more recently, chocolate milk, with EPA and DHA. This company prides itself for offering all the benefit of n-3s "without con-taminant concerns or sacrificin delicious taste." The company's all-natural n-3 is derived from purifie cod liver oil from fis in Norwegian Arctic waters. It is odourless and unde-tectable to the palate. Each serving of its products provides 75 mg of EPA and DHA. Another company recently announced that it has successfully solubilized fis oil and n-3 fatty acids (DHA/EPA/α-linolenic acid (ALA)) using a novel technology, which provides improved solubility, enhanced bioavailability, and particle size reduction to the nanometre level of water-insoluble lipophilic compounds. In brief, the process solubilizes n-3 by "reformulat-ing and improving" it. When PUFAs are appropriately mixed with polyoxyethanyl-alpha-tocopheryl sebacate (PTS), n-3 oils readily disperse in water resulting in translucent, stable aqueous solutions even at very high concentrations. Clarity is achieved through particle size reduction down to the nanometre level. There are many methods in the industry known to prolong sensory stability of PUFA microemulsions, but all have had only limited success. These include use of antioxidants, enzymes, complexing agents, emulsion surface engineer-ing, chemical derivatization, encapsulation, and fl vour masking, etc. Alternatively, particle size reduction (from classic micro-emulsions to PTS-enabled nanoparticles) dramatically changes the characteristics of the solutions they provide [24].

In addition, water-soluble/dispersible dried n-3 powders inherently enhance the product's stability. The value of a novel technology for consumer product applications has been further enhanced by obtaining GRAS status for PTS, the lead solubilizing compound in that tech-nology. Meanwhile, a Canadian company has captured the market for n-3 products because of its microencapsulated products that are not dissolved in the product until they reach the gastrointestinal (GI) tract. If the current trends continue, the future for n-3 beverages is most certainly bright.

Fish oils have shown potential clinical benefit and been used in health care as supplements and therapeuticals (Table 39.2). Increased intake of n-3 fatty acids reduces incorporation of arachidonic acid (AA) into cell membranes, thereby promoting a net anti-inflammator response. Indeed, fis oil has been shown to significantl decrease potent inflammator markers, including leukotrienes, prostaglandins, interleukins, and tumour necrosis factor. The inhibition of AA by n-3 fatty acids also produces a potential anti-thrombotic effect by decreasing production of thromboxane A2. Membrane stabilization in cardiac tissue by n-3 fatty acids confers potential anti-arrhythmic effects, whereas TAG lowering effects are caused by the reduction and secretion of very low-density lipoprotein (LDL) particles from the liver. With its unique and varied mechanisms of actions, the potential uses of fis oil have been studied in various clinical situations [26–28].

There is evidence from multiple studies that intake of recommended amounts of DHA and EPA in the form of dietary fis or fis oil supplements lowers TAG [29], reduces the

risk of death, heart attack, dangerous abnormal heart rhythms, and atrial fibrillatio [7], as well as strokes in people with known cardiovascular disease (CVD), slows the build-up of atherosclerotic plaques ("hardening of the arteries"), and lowers blood pressure in patients with marginally-high blood pressure [30]. However, high doses may have harmful effects, such as an increased risk of bleeding. Although similar benefit are proposed for ALA, scientifi evidence is still emerging, and beneficia effects may be less pronounced, due to limited conversion of ALA to its long-chain counterparts.

The benefit of fis oil in CVD provide the strongest and most compelling evidence favouring its use. With its potential anti-inflammator actions and membrane stabilization properties, fis oil supplementation has been studied in a wide spectrum of conditions. Studies in inflammator bowel disease, asthma, atopic dermatitis, psoriasis, renal disease, and psychiatric illnesses are either inconsistent or confine to limited studies. Studies in neurologic disorders such as ischemic stroke and Alzheimer disease are emerging [31,32].

Fish oil supplements provide the most consistent way of supplying higher doses of n-3 fatty acids. Supplementation can be considered as an alternative to dietary intake for persons who are averse to a fish-enriche diet and may also be lower in mercury content and other environmental pollutants.

Physicians can safely offer patients an alternative therapeutic approach for difficult-to-trea conditions such as hypertriglyceridemia and rheumatoid arthritis. For those without CVD, including young mothers and women, it is reasonable to recommend 650 to 1000 mg of low-risk fis oils per day through dietary approaches, as suggested by the National Institutes of Health working group, American Health Association (AHA), and the FDA – easily achieved by one to two fatty fis meals per week.

39.5 Conclusions

Marine oils from different sources may be processed into a variety of products from simply refine to encapsulated and microencapsulated as well as concentrates, both as simple alkyl esters and reconstituted TAG. Stability and stabilization of the oils would be important and essential in their use in products for food and supplement applications. For food applications, if used as such, they must be in products that are consumed within a short period of time and if microencapsulated they should remain in the intact form until reaching the GI tract for best performance in terms of fl vour and stability characteristics.

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40 Bioactive peptides from seafood and their health effects

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40.1 Introduction

"Food-derived bioactive peptides" have regulatory functions in the human system beyond their basic role as nutrient sources [1]. These peptides may be directly present in food, or can be released from different dietary proteins of plant or animal origin during gastrointestinal digestion, food processing, or by a fermentation process [2]. In recent years, seafood proteins, especially fis proteins, have gained much attention as an attractive source of bioactive peptides, due to the abundance of raw materials in the form of processing discards and underutilized species, in conjunction with research finding indicating an array of biological activities for fis protein hydrolysates (FPH) and specifi peptides derived from these hydrolysates [3,4]. The reported bioactivities for seafood-derived peptides include antihypertensive, antioxidative, immunomodulatory, neuroactive, hormone-regulating, antimicrobial, and mineral binding properties [5].

This chapter summarizes the value of seafood-derived bioactive peptides, by illustrating the sources of and methods for production of protein hydrolysates and specifi target peptides, and the diverse range of activities attributed to them. Examples of their applications as functional food ingredients and nutraceuticals for enhancing health are also discussed.

40.2 Sources of bioactive peptides from seafood

Hydrolysates and peptides produced by different means or inherently found in various seafood sources have been associated with a diverse range of bioactive properties, as illustrated in Table 40.1 [6–24] and further described below.

40.2.1 Enzymatic production of protein hydrolysates

Enzymatic hydrolysis has been the most common method of producing bioactive peptides from marine sources [2,4]. Biologically active peptides can be produced from precursor proteins using endogenous proteolytic enzymes already present in muscle or viscera of fis or shellfish and/or by adding exogenous enzymes from other sources [25]. Use of exogenous

Table 40.1 Examples of diversity of bioactive peptides from seafood

Method of preparation	Examples	Reported bioactivity	Reference
Enzymatic hydrolysis by exogenous enzymes	LKPNM from thermolysin digest of dried bonito	ACE-inhibitory ($IC_{50} = 2.4$ μM)	[6]
	Hydrolysate of Pacific hake (*Merluccius productus*) fish fillets using Protamex™	ACE-inhibitory ($IC_{50} = 165$ μg/mL)	[7,8]
	CgPep33 from oyster (*Crassostrea gigas*) using Alcalase® and bromelain	Antimicrobial	[9]
	NADPGLNGLEGLA and NGLEGLK from giant squid (*Dosidicus gigas*) muscle hydrolysate	Antioxidative	[10]
	Cod head and shrimp waste hydrolysates	Opioid, ACE-inhibitory	[11]
	VLSGGTTMAMYTLV from Alaska pollock (*Theragra chalcogramma*) using pepsin	Ca-binding	[12]
	VLSGGTTMYASLYAE from hoki (*Johnius belengerii*) frames using pepsin	Ca-binding	[13]
Enzymatic hydrolysis by endogenous enzymes	Sardine autolysate	Opioid	[11]
	LPHSGY from Alaska pollock (*Theragra chalcogramma*)	Antioxidative	[14]
	RPDFDLEPPY from yellowfin sole (*Limanda aspera*)	Antioxidative	[15]
	Hydrolysates from Pacific hake muscle with cathepsin L-like protease activity	Antioxidative	[16]
	Hydrolysates from North Atlantic cod, salmon and trout using digestive enzymes (pepsin and trypsin) or by autolysis	Inhibitory activity against prolyl endopeptidase	[17]
Fermentation	HFGBPFH from fermented mussel (*Mytilus edulis*) sauce	Antioxidative	[18]
	Asian fish sauce	Immunomodulatory	[19]
	Fermented sauces of oyster, bonito, anchovy, and sardine	ACE-inhibitory	[20,21]
Gastrointestinal (GI) digestion	LVGDEQAVPAVCVP from *in vitro* GI digestion of mussel (*Mytilus coruscus*) protein	Antioxidative	[22]
	LKP from GI digestion of LKPNM from dried bonito	ACE-inhibitory ($IC_{50} = 0.32$ μM)	[6]
Endogenous peptides	Carnosine, anserine, and ophidine	Antioxidative	[23]
	Protamines from Pacific herring (*Clupea pallasii*), chum salmon (*Oncorhynchus keta*), and rainbow trout (*Salmo irideus*), pleurocidin from the skin secretions of the winter flounder (*Pleuronectes americanus*), misgurin from mudfish (*Misgurnus anguillicaudatus*), pardaxin from Moses sole fish, callinectin from blue crab (*C. sapidus*), and panaeidins from Pacific white shrimp (*Panaeus vannamei*)	Antimicrobial	[24]

enzymes is preferred in most cases to the autolytic process due to the reduction in time required to obtain a similar degree of hydrolysis as well as better control of the hydrolysis to obtain more consistent molecular weight profile and peptide composition. Despite the potential problems, the advantages of lower cost have prompted use of endogenous enzymes in some cases to produce various protein hydrolysates with bioactive peptides (Table 40.1).

40.2.2   Formation of bioactive peptides by food processing and gastrointestinal (GI) digestion

Bioactive peptides can be liberated from precursor proteins during food processing including fermentation or by gastrointestinal (GI) digestion. In fermented fis and other seafood products, biopeptides are produced due to the action of microbes and endogenous proteolytic enzymes [25,26], while enzymes such as pepsin, trypsin, α-chymotrypsin, elastase, and carboxypeptidase A and B are involved during GI digestion [27]. A tetradecapeptide released from *in vitro* GI digestion of mussel (*Mytilus coruscus*) protein exhibited potent antioxidative activity, inhibiting the formation of reactive oxygen species from the peroxidation of polyunsaturated fatty acids (PUFA) [22, Table 40.1]. The angiotensin I converting enzyme (ACE)-inhibitory activity of two oligopeptides (IWHHT and IVGRPRHQG) from dried bonito increased upon incubation with chymotrypsin and trypsin, respectively [28].

40.2.3   Endogenous bioactive peptides from seafood

Carnosine (β-alanyl-*L*-histidine), anserine (β-alanyl-*L*-1-methylhistidine), and ophidine (β-alanyl-*L*-3-methylhistidine) are three antioxidative dipeptides naturally present in skeletal muscle tissues, in amounts that depend on the animal, its age, and diet [23]. Carnosine demonstrated both *in vivo* and *in vitro* antioxidative activity in rat skeletal muscle lipid and protein components under conditions of oxidative stress [29]. Vasodilatory action [30] and anti-ageing or anti-glycating property [31] of carnosine were also reported. Moreover, carnosine, anserine, and ophidine have been studied to determine potential physiological functions related to neurotransmitter synthesis [32]. Antimicrobial peptides are another class of peptides naturally present in many food sources, including fish crustaceans, and molluscs [24].

40.3   Potential health benefits of bioactive peptides derived from seafood

40.3.1   Antihypertensive peptides

Antihypertensive peptides may contribute to the lowering of blood pressure through inhibition of enzymes including ACE or endothelin converting enzyme (ECE) [33]. ACE-inhibitory peptides have been derived from various food sources including fish other marine animals, and algae [33,34]. Suetsuna and Osajima [35] were the firs to report on ACE-inhibitory peptides from fis muscle proteins, specificall from hydrolysates of sardine and hair tail meat prepared using denazyme AP (a protease from *Aspergillus oryzae*). Since then, ACE-inhibitory activity has been detected in an array of seafood protein hydrolysates and some fermented products, and in fact is the most studied biological activity of peptides derived from fis and other seafood. Furthermore, ACE-inhibitory peptide sequences have been identifie

from fis protein hydrolysates of bonito, anchovy, salmon, tuna, sardine, yellowfi sole, and Alaska pollock, as well as from other seafood sources such as mussel, squid, and oyster [33,34]. In fact, due to its promising antihypertensive effect according to animal and clinical studies [36], the oligopeptide LKPNM isolated from a thermolysin digest of the traditional Japanese dried bonito ("katsuobushi", Table 40.1) has been officiall approved for use under the Food for Specifie Health Use (FOSHU) category in Japan [34] and was file with the US Food and Drug Administration (FDA) as a new dietary supplement ingredient [37].

40.3.2   Antioxidative peptides

Even though there are few *in vivo* studies conducted to date, *in vitro* studies using various chemical assays and cell culture systems have proven the ability of these food-derived peptides to act as antioxidative agents to control various oxidative processes in food as well as potentially in the human body. Antioxidative potential of a hydrolysate made from capelin using Alcalase$^{®}$ was reported for the firs time by Amarowicz and Shahidi [38]. Jao and Ko [39] identifie seven antioxidative peptides from a tuna cooking juice hydrolysate with potential radical scavenging abilities towards 1,1-diphenyl-2-picrylhdrazyl (DPPH). Two peptides isolated from a giant squid muscle hydrolysate with high (>75%) content of hydrophobic amino acids, showed potential to act as chain-breaking antioxidants by inhibiting radical-mediated peroxidation of linoleic acid [10, Table 40.1]. Low concentrations (50 μg/mL) of these peptides could enhance viability of cytotoxic embryonic lung fibroblast cells, presumably by suppressing radical-induced oxidation of membrane lipids. The peptide HGPLGPL identifie from a tryptic hydrolysate of hoki skin gelatin showed strong radical scavenging activity and could also increase the antioxidative enzyme levels in cultured human hepatoma cells by helping to maintain the redox balance in the cell environment [40]. Furthermore, the peptide GPOGPOGPOGPOG purifie from gelatin hydrolysate of Alaska pollock skin also indicated a potent antioxidative activity [41].

40.3.3   Immunomodulatory peptides

The immunomodulatory potential of FPHs and fermented fis sauce has been reported, although further research is required to identify the peptides responsible for this bioactivity and to validate their effica y in humans. According to Thongthai and Gildberg [19], peptides in an Asian fis sauce had a stimulating effect in proliferating white blood cells in human subjects. Peptide fractions from Atlantic cod (*Gadus morhua* L.) stomach hydrolysate [42] and a muscle protein hydrolysate [43] with peptides having molecular weights ranging from 500 to 3,000 Da, have been reported to possess immuno-stimulatory activity, both *in vitro* and *in vivo*.

40.3.4   Neuroactive peptides

Peptides with opioid activities play an important role in functioning of the nervous system. Prolyl endopeptidase (EC 3.4.21.26) is an enzyme that cleaves proline-containing peptides, including neuropeptides, and inhibitors of this enzyme have been suggested as therapeutic agents for cognitive problems associated with aging or neurodegenerative diseases. Sørensen *et al*. [44] reported strong inhibitory activity against prolyl endopeptidase in the hydrolysates from three fis species (North Atlantic cod, salmon, and trout) by either hydrolysis with

digestive enzymes (pepsin and trypsin) or by autolysis. Opioid activities were also found in sardine and cod head hydrolysates [11].

40.3.5   Hormonal and hormone-regulating peptides

Peptides such as gastrin, hormone releasing factors (GRFs), and calcitonin gene related peptide (CGRP) identifie from marine sources exert complex and multiple physiological effects by serving as hormones themselves and/or by regulating hormonal responses associated with the control of important metabolic, growth, and development processes [45]. CGRP-related molecules have been purifie from cod hydrolysates from heads, stomach, and viscera, shrimp head hydrolysate, cooked sardine head, and gut hydrolysate, and from a cooked siki (*Centroscymnus coelolepsis*) head hydrolysate [46–48]. Purifie CGRP-like peptides from hydrolysates of both sardine (molecular weight of 6,000 Da) and siki (molecular weight around 1,500 Da) induced an inhibition of the CGRP-stimulated adenylate cyclase activity, suggesting that these molecules may act as antagonists for peptides that bind to CGRP receptors in rat liver membranes [47,48]. Beneficia effects of short-term or prolonged infusions of CGRP in patients to increase cardiac output and to decrease blood pressure without changes in heart rate have been described by some studies [49,50], while other studies have documented its role in the processing of auditive information and facilitation of learning and memory processing [51]. Furthermore, CGRP-like molecules have the capacity to exert gastric acid protection by decreasing acid secretion and by increasing the blood fl w [52].

40.3.6   Antimicrobial peptides

Examples of some naturally occurring antimicrobial peptides from marine sources are shown in Table 40.1. Among these, peptides such as misgurin and pleurocidin have the ability to disintegrate the phospholipid-rich microbial membrane, due to their cationic nature and α-helical structure. On the other hand, the mechanism of the antimicrobial peptide pardaxin involves interference of cell membrane ionic transport [24]. Apart from these naturally occurring antimicrobial peptides from various seafood sources, there may also be a potential to produce antimicrobial peptides using proteolytic enzymes. For example, Liu *et al*. [9] recently reported the use of Alcalase$^®$ and bromelain for production of a cysteine-rich antimicrobial peptide *CgPep33* from the oyster (*Crassostrea gigas*). This peptide was active against all tested Gram-positive and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, and *Staphylococcus aureus*) as well as fungi (*Botrytis cinerea* and *Penicillium expansum*), with IC_{50} values (the concentration at which the microbial activity is inhibited by 50%) ranging from 18.6 to 48.2 μg/mL [9]. Beyond their effect on micro-organisms, cationic antimicrobial peptides may also act as chemokines for immature dendritic cells and memory T-cells and serve as a bridge between the innate and adaptive immune systems in human [53]. They also have been found to play a role in the wound healing process [44].

40.3.7   Other bioactive peptides from seafood

Jung *et al*. [12] and Jung and Kim [13] isolated and characterized low molecular weight peptides with high calcium binding affinit from pepsinolytic hydrolysates of Alaska pollock backbone and hoki frame, respectively (Table 40.1). These peptides could solubilize similar contents of calcium as the casein phosphopeptides from milk. The authors, therefore,

suggested the possibility of using these peptide sources as novel nutraceuticals with high calcium bioavailability for oriental people with lactose indigestion and intolerance, for example, as calcium-fortifie supplements or calcium-enriched foods such as fruit juice as an alternative for dairy products [12,13]. Various other bioactive properties of FPH from seafood have been reported that await further research to identify the active peptides and possible mechanism of action. For example, an FPH from salmon frames administered to rats helped to reduce total cholesterol and increased HDL cholesterol levels [54], and FPHs have been reported to improve glucose tolerance and insulin sensitivity [55]. A mackerel protein hydrolysate was found to have a blood thinning effect [56], and a small protein fragment of 12.01 kDa molecular size with anticoagulant and antiplatelet properties was recently isolated from a yellowfi sole FPH [57]. FPH produced through enzymatic hydrolysis of Atlantic salmon (*Salmon salar*, L.) frame proteins, which was incorporated to form 20% of the crude protein in the diet of 4 to 5-week-old male rats, was claimed to have a potential for treatment and/or prevention of atherosclerosis, coronary heart disease (CHD), stenosis, thrombosis, myocardial infarction, stroke, and fatty liver [58].

40.4   Current and future applications

As described earlier, the effica y of using seafood-derived peptides for maintaining a lower blood pressure has been well established. Sardine protein hydrolysate is widely available as supplements for this purpose in Japan [59]. A vegetable drink with added sardine hydrolysate at a 0.5 g dose was helpful in lowering systolic blood pressure [60]. "Peptide Soup" is another product manufactured by Nippon in Japan that contains bonito-derived peptides and claimed to have a hypotensive effect [1]. Blood pressure lowering capsules (e.g. Peptide ACE 3000 by Nippon Supplement Inc.; Vasotensin 120T^{TM} by Metagenics, USA; PeptACE^{TM} Peptides 90 by Natural Factors, USA) have also been made with the Katsuobushi Oligopeptide LKPNM from thermolysin-digested dried bonito [1]. Seacure$^®$ is a commercially available fermented fis product made by controlled yeast fermentation of Pacifi whiting or Pacifi hake (*Merluccius productus*), which is claimed to be beneficia for a variety of gut conditions, such as the injurious effects of non-steroidal anti-inflammator drugs and other ulcerative conditions of the bowel [61]. Two FPHs that are derived from hydrolysis of cod (*Gadus*) and mackerel (*Scomber*) proteins and marketed as nutritional supplements (commercial names PC60 and Stabilium 200) have been found to reduce anxiety and also improve memory and learning in rats and patients [62]. Due to their vasodilatory action, incorporation of CGRPs isolated from various marine protein hydrolysates into functional foods might be an important defence mechanism against the serious consequences of increased plasma volume including edema, hypertension, and increased cardiac workload, and might also have therapeutic potential in the treatment of pathologies such as obesity or gastric ulcer [48]. Seafood-derived antimicrobial peptides may also have a great potential to increase the nutritional immunity and will be candidates in developing therapeutic agents for topical or systemic administration.

40.5   Conclusions

Food products, supplements, or natural health products containing bioactive peptides from seafood should be expected to command a huge market, given the many potential health benefit discussed above. However, few commercial products are available to date, which

may be attributed to a variety of reasons, including a lack of clinical trials confirmin bioactivity, effica y and safety, poor functional properties, high production cost, problems in making a reproducible product, and bitterness or other organoleptic problems. Strict regulations and lengthy procedures may also be involved to obtain approval for marketing the new product. Nevertheless, as discussed in Section 40.4, several successful products have been made incorporating seafood-derived bioactive peptides into functional foods and nutraceuticals, and the ever-increasing attention on consumer health will likely fuel greater demand for these types of products in the near future.

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41 Antioxidative properties of fish protein hydrolysates

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41.1 Introduction

Seafoods including fis are an important source of protein in the human diet, providing nearly 20% of the animal protein consumed by the world population [1]. Fish contains all the essential amino acids required for human growth and nutrition and hence constitutes an excellent source of nutritive and digestible protein [2]. Over the last several decades, an increasing demand and consumption of seafood products has lead to over-exploitation of world fis stocks. In addition to over-exploitation, large amounts of protein rich by-products are discarded without any attempt to recover them. If one merely looks at the processing by-products from filleting these raw materials may contain as much as 10 to 20% of fis protein and in many cases are not utilized for human or animal consumption. Possibly, more than 60% of fis tissue remaining after processing (species dependent) is considered as a processing waste and not used for human food.

In recent years, strict environmental regulations have been imposed, which no longer allow fis processors to discard their offal, resulting in high cost of refinin the material before it is discarded or directing it into low-grade fis meal or plant fertilizers [3]. Dwindling fis stocks and under-utilization of aquatic by-products has provoked a strong and urgent need to develop alternative methods for better utilization of fis and seafood by-products. However, in order to be accepted by the industry, these processes have to be economically feasible compared to discarding the by-products or using them for feed or fertilizer [4,5]. A number of methods to better utilize raw materials of aquatic origin have been proposed, one of them being enzymatic hydrolysis to produce what is collectively called fis protein hydrolysate (FPH).

The use of enzymes for predigesting food proteins has been used for centuries for tenderizing meat and for making products such as tempeh, tofu, fis sauce, and fermented herring. By applying enzyme technology to recover and modify fis proteins present in the by-products of fis processing, it is possible to produce a broad spectrum of protein ingredients with a wide range of food and nutraceutical applications [4,6]. This approach could make better use of by-products and at the same time be employed for under-utilized fis species, increasing the margin of profi for the fishin industry and creating a more environmentally friendly industry. Significan work on recovery of fis proteins was conducted in the 1960s

and 1970s [4,6–11] and was directed into the production of cheap nutritious protein sources for rapidly growing developing countries or towards animal feed production, primarily as fis protein concentrate, which employs chemical hydrolysis. However, over the last two decades, several researchers have reported the potential of using FPH in food formulations [4,6,8–11]. In recent years, there has been growing evidence that hydrolyzed fis proteins may have very high bioactivity, thus creating a new avenue for fis protein ingredients. In this chapter, the potential of FPH as a food antioxidant and bioactive ingredient to combat oxidative processes in living systems is discussed.

41.2   FPH as food antioxidants

Antioxidants are important both from the perspective of food products and for their implications on human health. In food, antioxidants are added to improve the quality and sensory attributes such as colour, fl vour, and texture. In human nutrition, antioxidants play an important role in promoting health and in preventing diseases. Lipid containing food products can undergo enzymatic or non-enzymatic oxidative rancidity [12]. Non-enzymatic oxidation is usually controlled by the addition of natural or synthetic antioxidants. Antioxidants can possess varying degrees of hydrophilic and hydrophobic characters [13], and depending on their nature, antioxidants can act from the aqueous phase or on the water-lipid interface. A wide variety of natural and synthetic compounds can be used as food antioxidants. Synthetic antioxidants such as propyl gallate and butylated hydroxyanisole (BHA), and natural antioxidants such as tocopherol, rosemary extracts, and fl vonoids are widely used in the food industry. When antioxidants are added to food products, the active antioxidant components are usually added in small quantities (parts per million levels). At these small levels, antioxidants may or may not have any influenc on human health.

During the last two decades, protein hydrolysates prepared from plant [14], dairy [15], and aquatic sources [16] have gained popularity as natural food antioxidants. The popularity of protein hydrolysates as food antioxidants stem from the concerns surrounding the adverse effects of synthetic antioxidants [17,18]. Due to dwindling fis stocks and the strong need to better utilize the by-products of the aquatic industry, researchers have started focusing on FPH as a potential source of antioxidant in food and living systems. As early as 1990, Hatate *et al*. [19] demonstrated that sardine FPH has antioxidative properties. Later studies by Shahidi *et al*. [16] demonstrated that FPH could function as antioxidants in food systems. The antioxidant nature of FPH may arise from their size, hydrophilic-hydrophobic balance, partitioning capability, nature of functional groups, amino acid composition, metal chelating ability, and radical scavenging ability. In the following sections, the antioxidant properties of FPH are discussed in more detail.

41.2.1   Effect of enzymes on antioxidative activity

The type of enzymes used for hydrolyzing fis proteins can play an important role in determining the antioxidant activity of FPH. Each enzyme and enzyme preparation has its unique activity and can produce different peptides which, in turn, can have different functions and properties. Endopeptidases cleave inside the protein molecule giving rise to relatively large peptides, while exopeptidases cleave amino acids from the end of the protein molecule, giving rise to a combination of amino acids as well as small and large peptides. Thiansalkul *et al*. [20] reported that for the same degree of hydrolysis, FPH prepared using
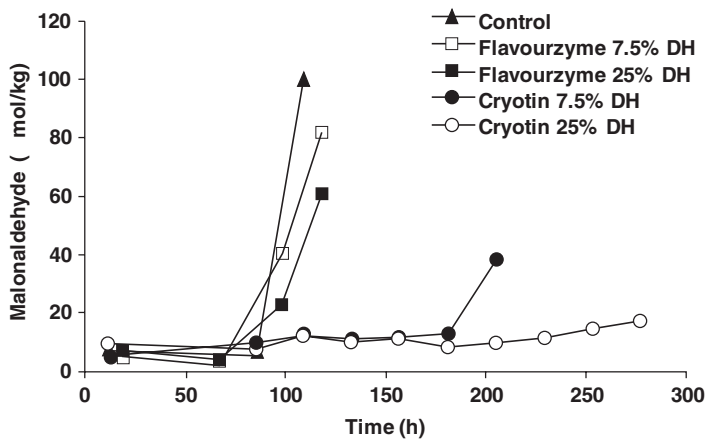
Fig. 41.1 Antioxidant ability of tilapia protein hydrolysates prepared using enzymes, Cryotin and Flavourzyme, at 7.5 and 25% degree of hydrolysis (DH). The hydrolysates were tested on washed tilapia model system for their ability to inhibit the formation of thiobarbituric acid reactive substances (TBARS). Oxidation was catalyzed using 12 mmol tilapia hemolysate. All hydrolysates were tested at same level of protein concentration. (Raghavan & Kristinsson [2009], unpublished.)

Flavourzyme (a mixture of endo- and exo-peptidase) exhibited higher radical scavenging ability and reducing power, but lower metal chelating ability compared to FPH prepared using Alcalase (an endopeptidase). In a similar study, Klompong *et al*. [21] determined that in yellow stripe trevally, FPH made using Flavourzyme were more antioxidative than those of Alcalase, indicated by higher DPPH radical scavenging ability, reducing power, and metal chelating ability. While studying tilapia FPH, Raghavan *et al*. [22] compared the antioxidant properties of different enzymes and found that hydrolysates prepared using Cryotin (a mixture of trypsin, chymotrypsin, and elastase) showed higher antioxidant activity in washed muscle systems, while hydrolysates prepared using Flavourzyme showed higher 2,2 diphenylpicrylhydrazyl (DPPH) radical scavenging ability (Figs 41.1 and 41.2). In FPH prepared from yellowfi sole, Jun *et al*. [23] found that hydrolysates prepared using pepsin
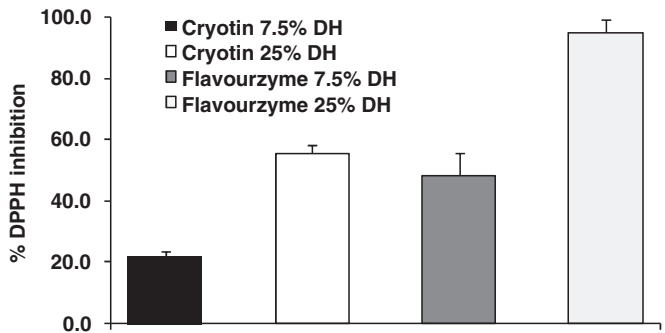
Fig. 41.2 Ability of tilapia protein hydrolysates prepared using enzymes, Cryotin and Flavourzyme, at 7.5 and 25% degree of hydrolysis (DH) to inhibit the formation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. All hydrolysates were tested at the same level of protein concentration. (Raghavan & Kristinsson [2009], unpublished.)

exhibited about 70% inhibition of linoleic acid (18:2 n-6) peroxidation. Similarly, pepsin hydrolysates prepared from the backbone of tuna are reported to have higher antioxidant activity than hydrolysates prepared using Alcalase, chymotrypsin or Neutrase [24]. These studies show the important role of enzymes on the antioxidative properties of FPH. However, no proper cause and effect relationship between the nature of enzymes and the antioxidant potential of FPH prepared using those enzymes have been fully established.

41.2.2   Size of peptides on antioxidative activity

Studies have shown that the antioxidative properties of FPH are significantl affected by their size. In FPH prepared from tilapia, Raghavan *et al*. [22] observed that the ability of FPH to inhibit lipid oxidation in a washed muscle system and their ability to scavenge DPPH radicals decreased with an increase in molecular weight (MW) of peptides, for example, 7.5% degree of hydrolysis (DH) < 15%DH < 25%DH. Among cod frame hydrolysates, Jeon *et al*. [25] reported a high antioxidant ability for less than 10 kDa FPH compared to less than 30 kDa FPH. In FPH prepared from yellowfi sole frames, Jun *et al*. [23] used high performance liquid chromatography (HPLC) to isolate the most potent antioxidant peptide of MW 13 kDa. Wu *et al*. [26] studied mackerel hydrolysates and reported that its reducing power increased with an increase in time of hydrolysis. However, they also found that the ability to inhibit linoleic acid oxidation increased up to 10 hours hydrolysis time and then decreased on further hydrolysis to 25 hours. They reported a peptide of MW 1,400 Da to possess the optimum antioxidant activity. Although most reports on FPH indicate an increase in antioxidant activity with a decrease in MW of peptides, there are also some studies showing otherwise. In yellow stripe trevally, for example, Klompong *et al*. [27] reported a decrease in DPPH radical scavenging ability and reducing power with an increase in %DH using Alcalase. However, the metal chelating ability increased with an increase in %DH. Low MW peptides may experience less steric hindrance and hence come together and chelate ions much more easily than large MW peptides. A higher antioxidant power of low MW peptides could also be contributed to the ability of these peptides to work across the water-lipid barrier and scavenge radicals commonly encountered in complex food systems such as muscle foods. However, in addition to the size of peptide, the nature and composition of peptides would also play an important role in determining their antioxidant effica y.

41.2.3   Composition of FPH

The composition of FPH can play an important role in determining their antioxidant activity. The amino acid make-up of peptides would in turn depend on:

a) nature of raw material, for example, skin, frames, and muscle;
b) type of enzymes used for hydrolysis; and
c) hydrolysis conditions and %DH.

In general, the amino acid composition of different fis species such as rainbow trout, Atlantic salmon, coho salmon, cherry salmon, and channel catfis has been reported to be very similar [28,29]. This composition could be altered by change in diet, but for general purposes, the change may not be very significant However, the nature of raw material would play an important role. For example, FPH obtained from skin collagen would be different from that of muscle tissues, since skin would be rich in amino acids such as glycine, proline,

and hydroxyproline [89]. More importantly, the type of peptide cleavage and the degree of hydrolysis would determine the composition of FPH and its antioxidative properties. Jun *et al*. [23] hydrolyzed yellowfi sole frames using digestive enzymes and then used HPLC to separate 10 *N*-terminal amino acid residues with optimum antioxidant activity, in the following sequence: arginine, proline, aspartic acid, phenylalanine, aspartic acid, leucine, glutamic acid, proline, and tyrosine (Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Tyr). In hydrolysates prepared from hound shark, Bougatef *et al*. [30] reported that a fraction rich in histidine, methionine, tyrosine, leucine, isoleucine, glycine, and arginine to be most antioxidative. Salmon protamine hydrolysate produced using pancreatin was reported to contain a dipeptide, Pro-Arg, with maximum radical scavenging and antioxidant activity [31]. The hydrophobic-hydrophilic nature of amino acids in FPH may also play a role in antioxidant activity. Dong *et al*. [32] reported a higher content of hydrophobic amino acids when silver carp protein was hydrolyzed with Alcalase than with Flavourzyme. They also observed a higher antioxidant activity for Alcalase FPH, which they attributed to its increased hydrophobicity and hence increased lipid solubility and ability to act in linoleic acid emulsions.

41.2.4 Inhibition of primary and secondary lipid oxidation products

Numerous researchers have studied the ability of FPH to control or inhibit the formation of primary and secondary products of oxidation in various food model systems, including muscle foods, linoleic acid emulsions, and washed muscle model systems. The primary products of lipid oxidation are the lipid hydroperoxides, which are usually measured as peroxide value (PV). The secondary products of oxidation will vary with the composition of lipids and include various aldehydes, epoxides, and epiodioxides [33]. The ability of FPH to control the formation of secondary lipid oxidation products is often measured in terms of thiobarbituric acid reactive substances (TBARS) and expressed as malondialdehyde content. The TBARS assay has its limitations in terms of reliability for fatty acids with less than three double bonds, and also interference from compounds such as sugars, oligosaccharides, and anthocyanins [90]. However, many researchers have tested FPH in muscle foods. As membrane lipids of muscle foods contain polyunsaturated fatty acids (PUFA), they are susceptible to oxidation and hence the TBARS assay can be useful to evaluate the antioxidant properties of FPH. Similarly, in linoleic acid model systems, researchers have tested the ability of FPH to suppress the formation of TBARS. Readers should exercise caution not to interpret the antioxidant property of FPH in terms of TBARS assay alone, but use the information along with other relevant antioxidant assays [34].

While studying liposomal systems, Klompong *et al*. [21] determined that protein hydrolysates at 200 ppm from the fis yellow stripe trevally significantl inhibited the formation of TBARS. Je *et al*. [35] showed that FPH from the frames of Alaska pollock exhibited antioxidative activity in linoleic acid model system. Among hydrolysates of different MW, the authors found that those with MW of less than 1 kDa were most effective in inhibiting oxidation. When FPH from yellowfi sole frames were used, Jun *et al*. [23] found that fractions of protein hydrolysate prepared using pepsin and mackerel intestine crude enzyme showed a strong antioxidative activity in a linoleic acid model system, both in inhibiting primary and secondary lipid oxidation products. They identifie the molecular mass of the most potent antioxidant fraction as 13 kDa. Similarly, with cod FPH, Jeon *et al*. [25] reported

that fractions below 5 kDa were most effective in reducing lipid oxidation in linoleic acid emulsion system. When FPH was studied in muscle food, Shahidi *et al*. [36] determined that capelin FPH added to minced pork could reduce the formation of TBARS in the product by 17.7 to 60.4% [36]. When capelin FPH was fractionated, both pro- and anti-oxidative fractions were found [37]. Fractions with higher MW were found to have more antioxidant activity. In washed muscle systems, Raghavan *et al*. [22] showed that FPH from alkali-treated tilapia protein isolates were effective in controlling peroxide values and TBARS compared to the control. Fractionated samples of tilapia FPH showed that high MW samples (>30 kDa) were more antioxidative than low MW samples. The antioxidative activity of peptides in FPH could arise from the ability of the imidazole ring in histidine to trap lipid radicals [38], as well as due to the potent hydrogen donating groups such as tyrosine [39].

41.2.5 Reducing power, radical scavenging, and metal chelating ability of FPH

Free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Hence, antioxidants are added to food products to quench reactive radicals and terminate the peroxidation chain reaction. Quenching of radicals is important for improving the quality and stability of food products. In protein hydrolysates, several amino acids such as tyrosine, methionine, histidine, lysine, and tryptophan can act as antioxidants [40]. Kawashima *et al*. [41] showed that dipeptides containing branched-chain amino acids had higher antioxidant activities than those with no branched chains and that *N*-terminal branched amino acid in dipeptides were preferable to *C*-terminal peptides for antioxidant activity. As FPH usually contain all of the above mentioned amino acids, they could potentially have good free radical scavenging ability.

Radical reactions are usually initiated by metal ions and haem proteins. Hence, chelation of transition metal ions is yet another way to control oxidation. Erickson *et al*. [42] indicated that the capability of histidine-related compounds to control lipid peroxidation could be due to their ability to coordinate with iron and form a complex, making iron unavailable for initiating oxidation. The reducing power or the ability of FPH to donate electrons and prevent oxidation of biological compounds is also used for evaluating antioxidant activity. While studying FPH from scad protein, Thiansilakul *et al*. [20] observed that hydrolysates prepared using Flavourzyme exhibited higher DPPH radical scavenging ability and reducing power, but lower metal chelating ability compared to hydrolysates prepared using Alcalase. They attributed these effects to the MW and the chemical composition of the different peptides. In catfis protein hydrolysates, Theodore and Kristinsson [43] found that DPPH radical scavenging ability increased with an increase in %DH and with reduction in peptide sizes. Raghavan *et al*. [22,44] studied the DPPH radical scavenging ability and reducing ability of different enzyme FPH and reported an increase in these properties with increase in %DH. However, they also reported no significan difference in metal chelating ability of FPH made using different enzymes [22]. The metal chelating activity of FPH can also be dependent on pH. Klompong *et al*. [21] reported that metal chelating activity of hydrolysates prepared using Alcalase and Flavourzyme decreased in high alkaline and acidic pH ranges ($P < 0.05$). The authors suggested that peptides might undergo conformational changes at high alkaline and acidic pH values, leading to the loss in their ability in chelating the metal ions. The same authors reported decreased reducing power of hydrolysates prepared using Alcalase, with an increase in %DH. In mackerel hydrolysates, Wu *et al*. [26] reported that

1.4 kDa fractions were more antioxidative than other MW fractions. In FPH from silver carp, Dong *et al*. [32] reported that low MW peptides possessed greater metal chelation ability than high MW hydrolysates.

41.3 Sensory attributes of FPH

In order to effectively use FPH as food antioxidants and nutraceuticals, they have to be palatable. Numerous researchers have studied the fl vour profile of FPH and their use as fl vouring components in food products. The taste characteristics of FPH has been described in many ways: bland [45], bitter [7,46], glutamate, and umami taste [47]. However, the penetration of FPH into the market as a fl vour ingredient has been slow, mainly owing to the bitterness and fishines problems associated with certain peptides in FPH [7], as well as problems due to oxidation and microbial spoilage. The fl vour and the odour of FPH would depend on a variety of factors such as the types of enzymes used for hydrolysis, processing conditions, presence of lipids, the size of peptide units, as well as on the composition of peptides.

41.3.1 Effect of hydrolysis on flavour

It has been reported that a limited amount of hydrolysis leads to an increase in bitterness, while extensive hydrolysis that gives small peptides and free amino acids can reduce greatly bitterness and sometimes can even improve the fl vour profile similar to monosodium glutamate and related nucleotides [48,49]. The increase in the bitterness of hydrolysates has been associated with the release of peptides containing hydrophobic residues [50], which in turn would interact with the taste buds to give a bitter taste [51–53]. However, extensive hydrolysis breaks these peptides into smaller units, decreasing the undesirable bitter taste. Yu *et al*. [54] reported an increase in the bitterness intensity of FPH with an increase in %DH. On the other hand, very extensive hydrolysis of lobster waste gave a product of superior quality and no bitterness, possibly due to a large amount of free amino acids [8] and fl vour enhancing nucleotides. Noguchi *et al*. [55] fractionated the low MW peptides from FPH and reported that peptides containing high ratios of glutamic acid residues would have fl vour activity resembling monosodium glutamate (MSG). A similar MSG-like effect was also reported when proteins in tuna cooking water was hydrolyzed [56].

41.3.2 Effect of enzymes on flavour profile

The degree of bitterness can vary with the type of enzyme used for hydrolysis. Hence the choice of enzyme is important to avoid bitterness in FPH. Usually, enzyme mixtures with a proper balance of endo- and exo-peptidase activities are recommended. Alcalase, an exopeptidase with a high preference for cleaving off hydrophobic amino acids is often used for preparing hydrolysates with low bitterness [57]. Flavourzyme from Novo Nordisk is another promising enzyme to limit the development of bitterness and at the same time gets good functional properties from FPH [4,6]. Hoyle *et al*. [7] found that herring FPH made with papain gave higher bitterness scores than FPH made with Alcalase, even though the papain samples gave lower %DH. Enzyme hydrolysis using Pronase was reported to produce less bitterness than hydrolysis using fici or bromelain [46,58,59], while cod hydrolysates

prepared using Alcalase were less bitter than those prepared using Flavourzyme [60]. In FPH from salmon frames, Liaset *et al*. [91] reported reduced bitterness when bacterial protease, Protamex, was used. Baek *et al*. [61] concluded that the enzyme, Optimase, was not only economical for making FPH from crayfis processing by-products, but also had the potential to produce quality seafood fl vour extracts. These and many other studies emphasize the importance of the type of enzymes used for hydrolysis and their effect on bitterness. Some studies have shown that bitterness and off-fl vours may not be just due to peptides present in FPH but could also be due to products of lipid oxidation. A study on mackerel hydrolysis demonstrated that bitterness development was well correlated with increased lipid oxidation [62]. In herring, defatted substrate (and hence lower lipid oxidation products) used for making FPH had much lower bitterness compared to the original fatty substrate [7]. This underlines the importance of substrate selection and quality of the starting material for FPH process, as already oxidized raw material will lead to more oxidation problems during processing of FPH. The authors have measured a variety of commercial FPH products as well as FPH products produced under controlled conditions in the laboratory, and fin that FPH, even from relatively lean fis can be highly oxidized and thus not palatable. Oxidation products can also have harmful effects *in vivo*, which is in contradiction to the goal of producing bioactive and healthy fis peptides. It is evident that to prepare antioxidant FPH is important to minimize oxidation during processing and storage of FPH. Lipid oxidation can develop during the hydrolysis process, likely as active pro-oxidants (e.g. haem released from haem proteins) are brought in close proximity to lipids (particularly membrane lipids). Reaction conditions, such as relatively high heat and oxygen incorporation (via stirring) also accelerate oxidation. Drying can also greatly increase the development of lipid; oxidation.

41.3.3 Processing techniques to reduce off-flavours and odours of FPH

Several processing techniques have been suggested to mask or reduce bitterness of hydrolysates, but few of them have been applied to FPH. Treating hydrolysates with activated carbon may partially remove bitter peptides [63]. However, this method can result in reduced yield and loss of nutritional properties [64]. Han *et al*. [65] showed that the addition of invert sugar and heat treatment improved the fl vour of FPH through a process called 'maturation'. Post-hydrolysis treatment of FPH with exopeptidases may also lower bitterness of the product [66]. Bitterness can also be reduced using extraction with organic solvents such as butanol [60,66] and ethyl alcohol [67], and using resins such as cholestyramine [60]. Another interesting approach is to make plastein out of FPH, a process that reverses hydrolysis with proteases such as pepsin and papain, for example, by rejoining the hydrolyzed fragments [68,69]. During the poly-condensation of the hydrolyzed units, new polypeptides are formed, which aggregate via hydrophobic associations, thereby masking bitterness and giving a product with unique functional properties. The gel-like product may fin use as filler in a variety of products [70]. This reaction has been shown to be useful to recover protein from extensively autolyzed fis silage [71]. Off-fl vours and odours due to lipid oxidation can be minimized using proper antioxidative strategies and process modification such as reduced temperature (by using low temperature adaptive enzymes), and operating at neutral or alkaline pH values to reduce the pro-oxidative effects of haemoproteins. Minimal oxygen exposure can also help reduce lipid oxidation.

41.4 Physiological and bioactive properties of FPH

Free radicals and oxidative stress could cause several debilitating disease conditions such as inflammation impaired immune system, cancer, Alzheimer's disease, and macular degeneration [72,73]. Dietary proteins and peptides including FPH can possess specifi biological properties, which could make them potential ingredients for functional and health promoting foods. The bioactive properties of these hydrolysates would depend on specifi amino acid composition and sequence. In the following section, we would look at some of the health promoting properties of FPH.

41.4.1 Antiproliferative activity and reparative role of FPH

The production of reactive oxygen species (ROS) in living systems causes oxidative stress and is associated with various disease conditions such as cancer and cardiovascular diseases [74,75]. Oxidative stress can be alleviated by the consumption of antioxidant-rich foods. Several plant antioxidants such as polyphenols [76] and soy peptides [77] have been explored for their ability to scavenge ROS. The ability of peptides to suppress oxidative stress depends on the type of enzymes used for hydrolysis, hydrolysis conditions, as well as the size and the amino acid sequence of peptides, and the uptake of FPH through the intestinal walls of the digestive tract. The antioxidative activity of amino acids [40], dipeptides [41], and tripeptides [78] has been extensively researched and documented. In one such study, Picot *et al*. [79] studied the *in vitro* antiproliferative activity of FPH on breast cancer cell lines. The authors reported that FPH from cod, plaice, and salmon exerted a significan antiproliferative effect at 1 g/L. Other studies [80,81] investigated the induction of apoptosis in human lymphoma cell line by peptide fractions separated from anchovy sauce. They reported apoptosis induced DNA fragmentation in the peptide treated cell lines indicated by an increase in caspase-3 and caspase-8 activities. From their results, the authors suggested that peptides from anchovy may have chemopreventive effects through the induction of apoptosis in cancer cells. The reparative role of FPH has also been studied on epithelial injury and repair [82]. In this study, the authors reported that FPH possesses both pro-migratory and pro-proliferative activity, and reduction in the degree of injury sustained in an *in vivo* rat gastric damaging model. Some of these effects were contributed to the high concentrations of glutamine and glutamine containing peptides in FPH [83,84].

41.4.2 Immunomodulatory role of FPH

Immunomodulators are classifie as substances that may enhance, stimulate, or inhibit immune responses. Immune responses could lead to oxidative bursts and the production of ROS. These free radicals are useful since they are produced by phagocytes in response to bacterial infections. However, under certain conditions, an excess of ROS could be produced leading to deleterious conditions such as inflammation FPH may affect the production of ROS and thus may act as an immunomodulator. For example, Gildberg *et al*. [85] prepared acid peptide fractions from spray dried, emptied cod stomach FPH, and studied their stimulatory role on leukocytes of Atlantic salmon. These researchers reported an elevated oxidative burst in leukocytes after 2 and 7 days of incubation when the peptides were added at a concentration of 1 to 25 µg/mL. Among cod muscle FPH, peptides of MW 500 to 3,000 Da were reported to be the most potent immunomodulator [86] among all the MW fractions. However, there

are also other studies that indicate little or insufficien protection by immunostimulatory FPH when species such as Atlantic cod fry [87] and juvenile coho salmon [88] were exposed to high levels of highly virulent pathogens such as *Vibrio anguillarum*. Raghavan *et al*. [44] studied the effect of tilapia FPH for their ability to quench radicals generated *in vitro* by phorbol myristate acetate (PMA) stimulated human monocytes. The study reported that Flavourzyme (exopeptidase) and Cryotin (mixture of trypsin, chymotrypsin, and elastase) hydrolysates could show increased ability to scavenge radicals with an increase in the %DH, although no significan difference was observed between the different enzymes.

41.5   Conclusions

FPH have been investigated for a long time, and recent years have seen a surge of new interest into these products. Research into the properties of FPH has shown a shift in the new millennium from functional properties to their bioactive properties. There are a growing number of studies, as demonstrated in this chapter, showing the great potential of FPH as an ingredient to combat oxidation in food systems as well as oxidative stress in animals and humans. Still we have a way to go to understand the mechanisms behind FPH activity and the focus of future work should be on *in vivo* trials and clinical trials to validate and document the effects of FPH products in living systems. This information is critical if FPH are to be promoted as natural products to prevent and combat many common diseases and conditions.

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42 Functional and nutraceutical ingredients from marine macroalgae

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42.1 Introduction

Accumulating scientifi evidence on the relationship between diet and health has demonstrated that food products with beneficia components can improve the state of well-being and reduce the risk of common diseases. A growing number of health conscious consumers have created a demand for food products with health promoting benefits Therefore, search for new ingredients with biological activity from natural resources is gaining great interest among researchers.

Marine macroalgae have a long history of use as food and folk medicine in Asia and are traditionally well-known for their versatile health benefits Marine algae are not only a rich source of dietary fibre proteins, vitamins, and minerals, but also contain a great variety of secondary metabolites with diverse biological activities, which cannot be found in terrestrial plants. Therefore, the potential application of seaweed as a source of functional additives with health beneficia properties is a new target for exploration. In recent years, a number of potent antioxidant compounds have been isolated and identifie from different types of edible seaweeds. In particular, phlorotannins (polyphloroglucinol phenolics) derived from brown algae have been shown to possess multiple physiological activities such as antioxidant, anticarcinogenic, antibacterial, anti-inflammator , and anti-allergic properties (Table 42.1). Other bioactive compounds of importance in macroalgae are sulphated polysaccharides, dietary fibre polyunsaturated fatty acids (PUFA), sterols, carotenoids, and α-tocopherol [1]. This chapter discusses recent investigations on functional and nutraceutical ingredients from marine algae, with emphasis on the biological activities of algal polyphenols and sulphated polysaccharides.

42.2 Functional and nutraceutical properties of polyphenols from marine algae

42.2.1 Occurrence and chemical structure of algal polyphenols

Marine macroalgae are a rich source of polyphenols. A series of phenolic compounds such as catechins (e.g. gallocatechin, epicatechin, and catechin gallate), fl vonols, and fl vonol

Table 42.1 Some examples of bioactive ingredients isolated from marine algae and their multifunctional properties

Bioactive ingredients	Algae species	Bioactivity	Reference
Phlorotannins	**Phaeophyceae:** *Ecklonia cava, Ecklonia kurome, Ecklonia stolonifera, Eisenia bicyclis, Eisenia Arborea, Fucus vesiculosus, Fucus spiralis, Fucus serratus, Ascophyllum nodosum, Sargassum kjellmanianum, Sargassum ringgoldianum, Sargassum siliquastrum, Ishige okamurae*	Antioxidant, anti-inflammatory, anti-allergic, anti-tumour, anti-diabetic, antibacterial, HIV-1 reverse transcriptase and protease inhibitory activities, chemoprevention against several vascular diseases	[8,16,17,19,45,46]
Sulphated polysaccharides	**Phaeophyceae:** *Laminaria japonica, Ecklonia cava, Ecklonia kurome, Undaria pinnatifida, Fucus vesiculosus, Fucus evanescens, Ascophyllum nodosum, Padina gymnospora, Dictyota menstrualis, Spatoglossum schroederi, Sargassum fusiforme, Sargassum thunbergii, Sargassum stenophyllum, Sargassum latifolium, Sargassum fulvellum* **Rhodophyceae:** *Gigartina skottsbergii, Chondrus ocellatus, Porphyra haitanesis, Gracilaria cornea, Grateloupia filicina, Grateloupia longifolia* **Chlorophyceae:** *Ulva pertusa, Ulva conglobata, Codium pugniformis, Codium cylindricum, Monostroma angicava, Monostroma latissimum, Monostroma nitidum*	Antioxidant, anticoagulant, anti-inflammatory, antiviral, antibacterial, anti-tumour, antivasculogenic, antithrombotic, immunomodulatory, radioprotective activities	[21,23,24,26–30, 47,48]
Fucoxanthin	**Phaeophyceae:** *Hijikia fusiformis, Undaria pinnatifida, Laminaria japonica*	Antioxidant, anticarcinogenic, anti-obesity, anti-diabetes, anti-inflammatory activities	[33–35,37,38]
Sterols	**Phaeophyceae:** *Pelvetia siliquosa, Sargassum carpophyllum, Sargassum muticum, Sargassum parvivesiculosum, Ecklonia stolonifera* **Chlorophyceae:** *Ulva lactuca*	Antioxidant, anti-diabetes, anti- inflammatory, anti-tumour, antibacterial, nhibition in Alzheimer's disease	[40,41,49]
Bioactive peptides	**Phaeophyceae:** *Uradaria pinnatifida, Sargassum carpophyllum, Sargassum fulvellum, Sargassum horneri, Sargassum coreanum, Ishige okamurae, Ecklonia cava* **Rhodophyceae:** *Porphyra yezoensis* **Chlorophyceae:** *Chlorella pyrenoidosa*	Antihypertensive, antioxidant, antimicrobial, mineral-binding, immunomodulatory, anticarcinogenic, antiviral, opioid activities	[42–44]
PUFA (n-3)	**Phaeophyceae:** *Undaria pinnatifida, Hizikia fusiforme, Himanthalia elongata, Laminaria ochroleuca* **Rhodophyceae:** *Porphyra spp., Palmaria stenogona, Polysiphonia urceolata, Pachymeniopsis lanceolata, Gelidium amansii, Chondria crassicaulis* **Chlorophyceae:** *Ulva fenestrate*	Anti-inflammatory, anti-tumour, antiviral, antimicrobial activities, prevention of coronary heart disease, thrombosis, atherosclerosis	[1,50,51]

glycosides have been identifie from methanol extracts of red and brown algae [2–4]. Phlorotannins, the largest group of polyphenols in marine brown algae, exhibit many interesting physiological activities (Table 42.1). They have been identifie from *Ecklonia cava, Ecklonia stolonifera, Eisenia bicyclis, Sargassum kjellmanianum*, *Sargassum ringgoldianum*, *Fucus vesiculosus*, *Fucus serratus*, and *Ascophyllum nodosum*.

Based on the type of structural linkages between the phloroglucinol sub-units (diphenylethers or biphenyls) and on the number of additional hydroxyl groups, phlorotannins can be systematically classifie into six major subclasses: phlorethols, fucols, fuhalols, fucophlorethols, isofuhalols, and eckols [5,6]. The chemical structures of common algal phlorotannins are presented in Fig. 42.1.

Fucols are phlorotannin polymers in which the phloroglucinol units are connected only by C–C (aryl–aryl) bonds. The phloroglucinol units in phlorethols are linked only by C–O–C (aryl–ether) bonds. Fucophlorethols contain both biaryl and aryl–ether linkages. Fuhalols are connected exclusively via ether bonds. Eckols are characterized by the occurrence of at least one three-ring moiety with a dibenzo-1,4-dioxin unit substituted by a phenoxyl group at the C-4 position. Isofuhalols and endofucophlorethols are small, specialized groups isolated from specifi algal genera. Moreover, some phlorotannins can also be sulphated or halogenated [5].

42.2.2   Antioxidant activity of algal polyphenols

42.2.2.1   *In vitro antioxidant properties of algal polyphenols*

Different species of seaweed display varying degrees of antioxidant activity. Brown seaweeds generally show better antioxidant capacities than green and red seaweeds. In particular, some species such as *F. vesiculosus*, *E. cava*, and *S. ringgoldianum* have been reported to possess remarkably high antioxidant potential. Fifty percent ethanol extracts of 25 common seaweed species from the Japanese coast were screened for their antioxidant activities [7]. The highest radical scavenging effect was obtained for *S. ringgoldianum*. The chemical structure of the phlorotannin was identifie as a bifuhalol oligomer based on matrix-assisted laser desorption/ionization time-of-fligh mass spectrometry (MALDI-TOF MS) analysis. The partially purifie phlorotannin-rich fraction exhibited significan scavenging potencies on superoxide anion radicals, which were around fi e times higher than that of catechin. Recently, the authors studied the potential antioxidant activities of water and 70% acetone extracts from ten species of Icelandic seaweeds [8]. The results of this screening experiment showed that the different seaweed species contained different levels of total phenolics and possessed diverse antioxidant properties. Three fucoid species (*F. vesiculosus, F. serratus*, and *A. nodosum*) exhibited the greatest scavenging activities against 2,2-diphenyl-1-picrylhydrazyl (DPPH) and peroxyl radicals as well as considerable ferrous ion-chelating abilities.

Previous studies have shown that phenolic compounds are the main contributors to the antioxidant activity of various seaweeds. A positive correlation has been well documented between total phenolic content (TPC) and antioxidant activity of different seaweed extracts by many researchers. However, some studies reported poor or low correlation coefficient between TPC and antioxidant activities of several seaweed extracts. Other active compounds, such as fucoxanthin and sterols in solvent extracts and water-soluble sulphated polysaccharides, proteins or peptides, ascorbic acid, and glutathione (GSH) in water and enzymatic extracts, could also contribute partially to the overall antioxidant activity.
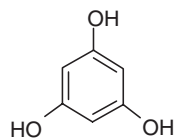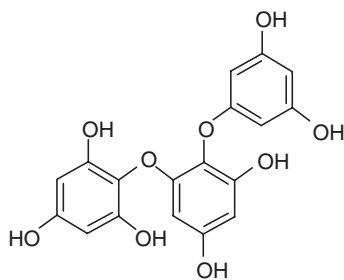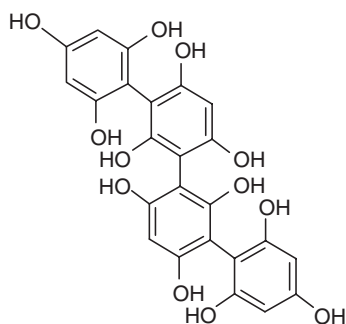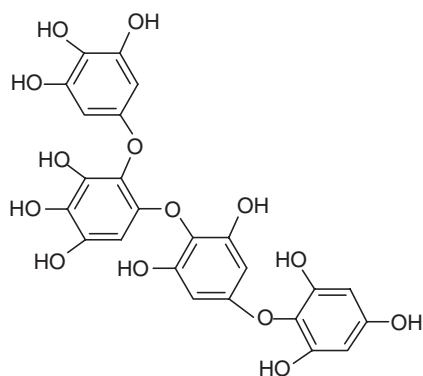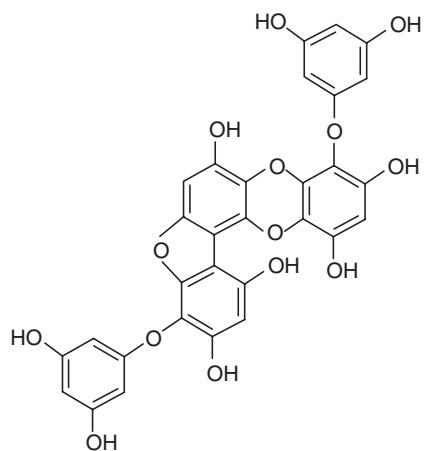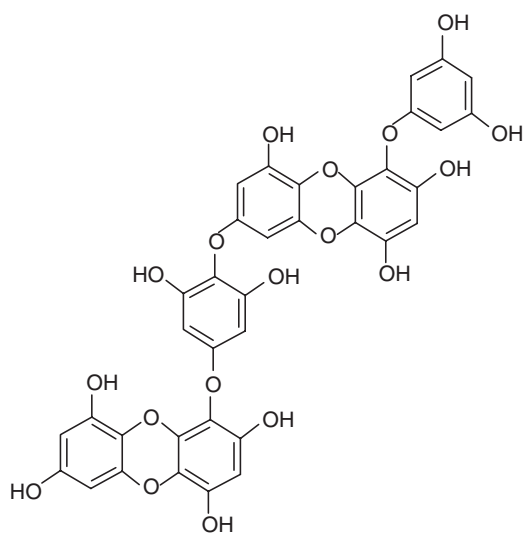
Fig. 42.1   Chemical structures of common algal phlorotannins.

42.2.2.2 *Antioxidant mechanism and structure-antioxidant activity relationship of algal polyphenols*

Plant and algal derived phenolic compounds have been shown to act as potent antioxidants in various systems, although some plant phenolics have sometimes been found to have pro-oxidant properties under certain conditions [9–10]. However, neither the antioxidant mechanism nor the structure-activity relationship of algal polyphenols has been fully elucidated up to date. The putative mechanisms have been associated with free radical scavenging, singlet oxygen quenching, transition metal ion chelation, and reducing power.

Many studies have demonstrated that phenolic compounds are potent free radical scavengers [9,11]. However, there are contradictory reports in the literature regarding metal chelating abilities of polyphenols. Some studies have shown that polyphenols derived from brown algae are potent ferrous ion chelators [12,13]. In contrast, other authors have reported that metal chelation played a minor role in the overall antioxidant activities of several plant-derived phenolic compounds [14]. In agreement with this, our study on various seaweeds using different antioxidant assays indicated that ferrous ion-chelating ability of seaweed extracts correlated neither with TPC nor with DPPH radical scavenging activity or oxygen radical absorbance capacity (ORAC). Therefore, the major role of algal polyphenols appeared to be as potent radical scavengers and primary, chain-breaking antioxidants [8]. Other components such as polysaccharides, proteins, or peptides may be more important for the observed chelating effects of the extracts.

Several studies have reported that high molecular weight (HMW) phlorotannins exhibit more potent antioxidant activities than the monomer phloroglucinol. It was postulated that oligomerization of phloroglucinol may be crucial for the enhancement of the radical scavenging activity. However, no clear correlation was found between the antioxidant activity and structural characterization of the polymer.

Yan *et al*. [15] reported that HMW phlorotannins from *S. kjellmanianum* were more effective than those of their low molecular weight (LMW) counterparts to prevent rancidity in fis oil. The antioxidant activity of HMW phlorotannins at a concentration of 1% was about 2.6 times higher than that of 0.02% butylated hydroxytoluene (BHT).

Recently, Shibata *et al*. [16] isolated and identifie several phlorotannins, including eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol from the Japanese Laminariaceous brown algae (*E. bicyclis*, *E. cava*, and *Ecklonia kurome*). All phloroglucinol oligomers displayed potent DPPH radical scavenging activity and were approximately twice as effective as catechin, ascorbic acid, and α-tocopherol. With the exception of eckol, all phlorotannins exhibited extraordinary superoxide anion radical scavenging ability, which were around 2 to 10 times more effective than ascorbic acid and α-tocopherol. The varying radical scavenging properties of phlorotannin polymers may be contributed by the phenolic hydroxy groups attached to the eckol skeleton.

42.2.3 Other biological activities of algal polyphenols

Recent studies have revealed that algal polyphenols, especially phlorotannins derived from brown algae not only exhibit potent antioxidant activities but also possess many other biological activities, including anti-inflammator , anti-allergic, anti-tumour, anti-diabetic, antibacterial, HIV-1 reverse transcriptase, and protease inhibitory activities as well as chemoprevention against several vascular diseases (Table 42.1). Their multiple physiological activities offer

many advantages for potential applications in nutraceutical, pharmaceutical, and cosmetic industries.

42.2.3.1 Angiotensin I-converting enzyme (ACE) inhibitory properties of algal polyphenols

Jung *et al*. [17] studied the ACE inhibitory properties of ethanol extracts from 10 edible Korean seaweeds, including fi e Phaeophyceae (*E. stolonifera*, *E. cava*, *Pelvetia siliqu-osa, Hizikia fusifome*, and *Undaria pinnatifid*), four Rhodophyceae (*Gigartina tenella, Gelidium amansii*, *Chondria crassicaulis*, and *Porphyra tenera*) and one Chlorophyceae (*Capsosiphon fulvescens*). *E. stolonifera* and *E. cava* possessed the highest inhibitory activities. Six phlorotannin compounds were further isolated from *E. stolonifera*. The compounds phlorofucofuroeckol A, eckol and dieckol, showed remarkably high inhibitory activities, whereas phloroglucinol showed no activity within the tested concentrations (163.93 µg/mL). Although the structure–activity relationship of the phlorotannins has not yet been established, a closed-ring dibenzo-1,4-dioxin moiety in the molecular skeleton seems to be crucial for their ACE inhibitory properties. Moreover, the presence of another dibenzofuran ring may also enhance the inhibitory effects.

42.2.3.2 Human immunodeficiency virus (HIV) inhibitory properties of algal polyphenols

A large-scale screening experiment was carried out to study the inhibitory activities of 47 types of Korean seaweeds on HIV type 1 (HIV-1) reverse transcriptase (RT) and HIV-1 integrase (IN) [18]. One of the 4 Chlorophyceae, 8 of the 17 Phaeophyceae, and 6 of the 26 Rhodophyceae showed inhibitory activities against HIV-1 RT. Five brown algae possessed inhibitory effects on the $3'$-processing activity of HIV-1 IN. In particular, the ethyl acetate (EtOAc) fraction of *E. cava* strongly inhibited both the HIV-1 RT and IN activities. In another study, they further isolated four phlorotannin compounds from the EtOAc fraction of *E. cava* and evaluated their inhibitory activities against HIV-1 RT and protease [19]. The compounds $8,8'$-bieckol and $8,4'''$-dieckol had strong inhibitory effects on the HIV-1 RT activity, whereas eckol and phlorofucofuroeckol A did not exhibit anti-HIV-1 RT activity. Furthermore, the inhibitory activity against HIV-1 RT of $8,8'$-bieckol was 10-fold higher than that of $8,4'''$-dieckol. The difference in the inhibition potential of these two compounds appears to be related to the steric hindrance of the hydroxyl and aryl groups near the biaryl linkage of $8,8'$-bieckol.

42.3 Functional and nutraceutical properties of sulphated polysaccharides from marine algae

Over the past decade, many studies have demonstrated that the sulphated polysaccharides from marine algae possess excellent biological properties, including antioxidant, anticoagulant, anti-inflammator , antiviral, antibacterial, anti-tumour, antivasculogenic, antithrombotic, immunomodulatory, and radio-protective activities (Table 42.1). Particularly fucoidans, a unique class of sulphated fucans, have been extensively studied due to their diverse biological activities. They have been isolated mainly from several orders of brown

algae (Phaeophyceae) such as Fucales and Laminariales, but also from Chordariales, Dictyotales, Dictyosiphonales, Ectocarpales, and Scytosiphonales [20].

42.3.1 Antioxidant activity of sulphated polysaccharides

Sulphated polysaccharides derived from *Laminaria japonica*, *E. kurome*, *F. vesiculosus*, and *Ulva pertusa* have been found to possess excellent antioxidant potency. Due to their HMW and heterogeneous structures, the correlation between the structure and antioxidant activity of sulphated polysaccharides has not been fully characterized. However, their antioxidant activity seems to be highly related to several structural parameters such as the degree of sulphation (DS), the sulphation position, the molecular size, monosaccharide composition, and glycosidic branching. Type of linkage and molecular geometry are also involved in exhibited antioxidant activity [21].

Tsipali *et al.* [22] compared the free radical scavenging activities of glucan and nonglucan polymers. Phosphated and sulphated glucan exhibited higher antioxidant potentials than glucan and other neutral polysaccharides. Moreover, the monosaccharide constitution also has an impact on the free radical scavenging activity of a variety of carbohydrate polymers. When the monosaccharides were arrayed in a polymer molecule, enhanced antioxidant ability was observed [22].

Qi *et al.* [21] reported that polysaccharides from *U. pertusa* with high sulphate content and LMW exhibited stronger reducing power and radical scavenging activities than other sulphated polysaccharides. Zhao *et al.* [23] also observed that LMW sulphated polysaccharides (8,000–10,000 Da) from brown algae, *L. japonica* possessed high scavenging abilities against superoxide, hydroxyl, and hypochlorous acid radicals. More recently, they further demonstrated that the chemical composition such as glucuronic acid and fucose content could also influenc the scavenging effects [24].

42.3.2 Other functional properties of sulphated polysaccharides

42.3.2.1 *Anticoagulant activities of sulphated polysaccharides*

Anticoagulant activities are one of the most intensely studied properties of sulphated polysaccharides. Sulphated polysaccharides derived from marine algae are effective coagulation modulators and have the potential to be used as alternatives to heparin, a conventional anticoagulant drug. Different sulphated polysaccharides with potent anticoagulant activities have been isolated from several brown algae such as *F. vesiculosus*, *A. nodosum, Padina gymnospora*, *Dictyota menstrualis*, and *Spatoglossum schroederi*, red algae *Gigartina skottsbergii*, and green algae *Codium cylindricum, Ulva conglobata, Monostroma nitidum*, and *Monostroma latissimum* (Table 42.1).

Fucoidans have been reported to possess powerful *in vitro* and *in vivo* anticoagulant properties, which have many advantages over cattle-derived heparin. Fucoidans purifie from *F. vesiculosus* and *A. nodosum* have been patented as anticoagulant drugs. The anticoagulant activities of fucoidans can be attributed to direct fucan-thrombin interaction and are highly related to DS and MW. Colliec *et al.* [25] observed that the anticoagulant activity of fucoidan was reduced with a decrease in the MW. A HMW fraction (58 kDa) was found to possess rather high thrombin inhibitory property, while a LMW of 21 kDa only showed moderate activity. The anticoagulant activity of fucoidan is believed to be mediated by heparin cofactor

II and/or antithrombin III. Fucoidan also enhances plasma clot lysis and the activation of plasminogen by tissue-type plasminogen activator (t-PA) or urokinase.

Sulphated polysaccharides isolated from several *Monostroma* species (Chlorophyceae) also show potent anticoagulant activities. Two different sulphated polysaccharides from *M. latissimum* and *M. nitidum* have been reported to exhibit stronger antithrombin activities than that of heparin or dermatan sulphate [26]. Recently, Zhang *et al*. [27] compared the thrombin inhibitory effects of a sulphated polysaccharide from *M. latissimum* and its fragments with different MW. The MW had profound influenc on the anticoagulant activity. A relatively longer saccharide chain length is required for better thrombin inhibition. The sulphated polysaccharides with high sulphate content have also been found to be desired for anticoagulant activity.

42.3.2.2 *Anti-tumour activities of sulphated polysaccharides*

Sulphated polysaccharides from marine algae have been found to have potent anti-tumour activities as well as lower side-effects. These sulphated polysaccharides including fucoidan isolated from brown algae *L. japonica*, *U. pinnatifid* , *Sargassum thunbergii*, and *Fucus evanescens*, λ-carrageenan polysaccharides from red algae *Chondrus ocellatus*, and κ-carrageenan oligosaccharides from *Kappaphycus striatum*. However, contradictory reports can be found in the literature regarding the biochemical and molecular principles of these anti-tumour polysaccharides. Some studies showed that only HMW fractions (MW >16,000 Da) of partially hydrolyzed glucan polysaccharides had anti-tumour activity. In contrast, other authors reported that the anti-tumour activity was dependent upon their basic structure-oligosaccharide unit. The heptasaccharides present in the polysaccharides exhibited even higher activity than the polysaccharides themselves [28]. The study conducted by Yuan and Song [29] showed that the MW had great impact on the anti-tumour activity of carrageenan polysaccharides. Degradation of *kappa*-carrageenan polysaccharides into oligosaccharides could enhance the biological activity. The tumour inhibitory activities of carrageenan oligosaccharides may be attributed to their recognition or interaction with the tumour-specifi oligosaccharide molecules. Further studies are needed to elucidate the relationship between structural features and anti-tumour activities.

42.3.2.3 *Antiviral activities of sulphated polysaccharides*

Sulphated polysaccharides derived from different brown and red algae have been demonstrated to possess remarkable antiviral activities against viruses responsible for human infectious diseases, including herpes simplex virus (HSV) types 1 and 2, respiratory syncytial virus (RSV), human cytomegalovirus (HCMV), influenz virus, and bovine viral diarrhoea virus. Furthermore, most of the algal polysaccharides have very low cytotoxic activities towards mammalian cells, which represent a potential new source for the development of safer antiviral agent.

Sulphated polysaccharides obtained from different brown algae such as *Caulerpa* spp., *Corallina* spp., *Hypnea charoides*, *Padina arborescens*, and *Sargassum patens* have shown potent antiviral activities against HSV types 1 and 2 with low levels of cytotoxicity. Particularly, fucoidans have demonstrated an extraordinary antiviral activity towards HSV types 1 and 2, RSV, and HCMV. The antiviral mechanism seems to be related to their inhibition effects on the binding of the viral particle to the host cell [30]. Other sulphated polysaccharides from red algae, such as galactan sulphate and sulphated xylomannan, are also good
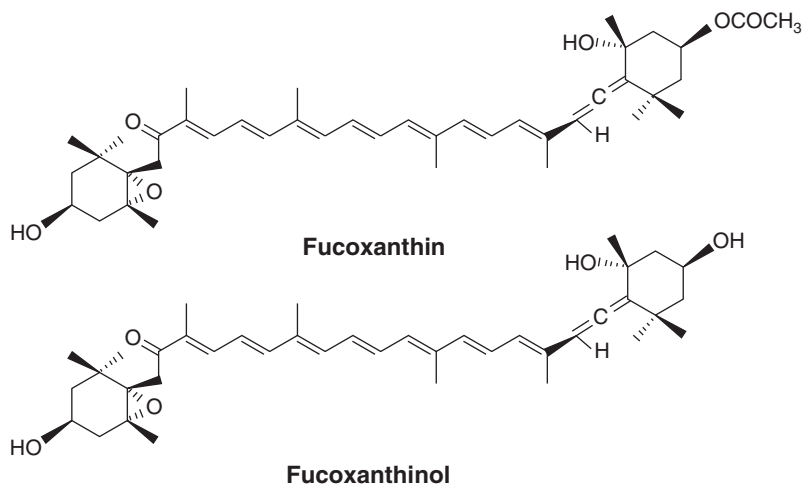
Fig. 42.2 Structure of fucoxanthin and fucoxanthinol.

candidates for further development of novel antiviral drugs. These polysaccharides have been found to be active during the initial stage of the RNA virus replication when the virus adsorbs onto the surface of the host cell [30].

42.4 Functional and nutraceutical properties of fucoxanthin from marine algae

Fucoxanthin, along with β-carotene, is one of the most abundant carotenoids found in nature. In marine macroalgae, high concentrations have been found in several edible brown algae such as *Hijikia fusiformis*, *U. pinnatifid* , and *Sargassum fulvellum*. However, it is absent in green and red algae. Fucoxanthin has a unique structure including an unusual allenic bond and 5,6-monoepoxide in its molecule [31] (Fig. 42.2). It can easily be converted to fucoxanthinol in human intestinal cells and mice. Therefore, fucoxanthinol may be the active form in the biological system [32]. Recently, the potential application of fucoxanthin in pharmaceutical and nutraceutical field has attracted an increasing interest due to its multi-functional properties including antioxidant, anticarcinogenic, anti-obesity, anti-diabetes, and anti-inflammator activities (Table 42.1).

42.4.1 Antioxidant activities of fucoxanthin

Similar to other carotenoids, fucoxanthin derived from brown algae is well-known for its antioxidant properties. Yan *et al*. [33] reported that the major carotenoid with DPPH radical scavenging activities in brown alga *H. fusiformis* was all-trans-fucoxanthin. The quenching of free radicals by fucoxanthin might stem from its ability to donate electron. More recently, a comprehensive study was carried out to evaluate the antioxidant activities of fucoxanthin and its metabolites, fucoxanthinol and halocynthiaxanthin [34]. The radical scavenging and singlet quenching activities of fucoxanthin and fucoxanthinol were comparable or even superior to that of α-tocopherol. Interestingly, fucoxanthin exhibited considerable DPPH

radical scavenging activity, even under anoxic conditions, whereas other carotenoids including β-carotene, β-cryptoxanthin, zeaxanthin, and lutein generally had no activities [35]. The superior radical quenching ability of fucoxanthin under anoxic conditions may be attributed to the presence of six oxygen atoms in its molecular skeleton, which ensures better interaction with free radicals. This distinct property is of great importance for the development of novel fucoxanthin-based antioxidant agent, since most tissues under physiological conditions generally have low oxygen presence.

42.4.2   Anti-obesity effects of fucoxanthin

The seaweed carotenoid, fucoxanthin, has recently been found to possess potent anti-obesity effects [36]. Feeding with fucoxanthin from edible brown algae *U. pinnatifid* significantl reduced the weight of abdominal white adipose tissues (WAT) of both rats and mice [37]. The mechanism of their anti-obesity properties may be related to the up-regulation of the expression of the fat-burning protein UCP1 (uncoupling protein 1 or thermogenin) in WAT around the internal organs [38]. Fucoxanthin-induced expression of UCP1 in WAT results in the oxidation of fatty acids and heat generation, which directly reduce abdominal fat in animals [39].

42.5   Functional and nutraceutical properties of sterols from marine algae

Sterols are another class of interesting constituents of marine algae. Brown algae generally contain higher level of sterols than red algae. Fucosterol and fucosterol derivatives are the predominant sterols of brown algae. The major sterol in red algae is cholesterol. However, some species contain mainly demosterol or 22-dehydrocholesterol. Sterol composition of green algae is much more complex and varied. Isofucosterol, cholesterol, chondrillasterol, poriferasterol, and ergosterol have been found to be the major sterols in various green algae. In recent years, sterols isolated from marine algae have been reported to exhibit diverse biological properties including antioxidant, anti-diabetes, anti-inflammation anti-tumour, antibacterial, and inhibition in Alzheimer's disease (Table 42.1).

42.5.1   Antioxidant activities of sterols from marine algae

In a study on antioxidant activities of fucosterol from brown algae *P. siliquosa*, it was observed that the serum transaminase activity was significantl decreased by fucosterol in CCl_4-intoxicated rats [40]. It also induced an increase in the activities of different antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase. Therefore, fucosterol not only possesses antioxidant, but also hepatoprotective properties.

42.5.2   Anti-diabetic activities of sterols from marine algae

Fucosterol isolated from *P. siliquosa* was found to exhibit potent anti-diabetic potential [41]. The hypoglycaemic effects of fucosterol is possibly due to the inhibition of hepatic glycogen breakdown in the liver, the reduction of hepatic glyconeogenesis, and the enhanced peripheral

glucose consumption or the direct inhibition of insulin release in the liver. Further studies are needed to elucidate the exact mechanisms of action.

42.6 Functional and nutraceutical properties of bioactive peptides from marine algae

Over the years, many efforts have been made for the isolation, characterization, and identifica tion of biologically active peptides from various food sources or protein hydrolysates. These peptides have been found to possess multifunctional properties including antihypertensive, antioxidant, antimicrobial, mineral-binding, immunomodulatory, anticarcinogenic, antiviral, and opioid activities (Table 42.1). In this chapter, we have focused on the antihypertensive activities of the peptides derived from marine algae.

42.6.1 Antihypertensive effects of the peptides derived from algae

Most peptides with antihypertensive activities are those derived from different food proteins and processing by-products. Seaweeds have also proven to be good sources of antihyperten-sive peptides. In particular, peptides derived from the brown seaweed *U. pinnatifid* (wakame) and the red algae *Porphyra yezoensis* have been demonstrated to be potent inhibitor of ACE [42–43]. Controlled enzymatic hydrolysis is one of the most appropriate methods in the production of tailor-made peptides from parent protein with enhanced ACE inhibitory prop-erties. An earlier study conducted by Suetsuna and Nakano [44] showed that the peptides from peptic hydrolysate of wakame had marked ACE inhibitory and antihypertensive activi-ties. Oral administration of these peptides significantl decreased the systolic blood pressure of spontaneously hypertensive rats (SHR). However, peptides of peptic digest of wakame showed pronounced bitter taste, which limited their use as functional food ingredients. In another screening test, the authors compared the antihypertensive effects of 17 different pro-tease hydrolysates of wakame, among which the hydrolysate made with protease S 'Amano' exhibited marked antihypertensive effect in both the single oral administration and long-term feeding test to SHR [42]. Moreover, S 'Amano' was more effective than other enzymes to produce a hydrolysate with improved sensory properties and high solubility in water.

42.7 Conclusions

Numerous scientifi studies across many laboratories have demonstrated that marine algae contain a great variety of components with diverse and unique biological activities, which cannot be found in terrestrial plants. The claimed physiological activities include antioxi-dant, anticarcinogenic, anti-vasculogenic, antithrombotic, anti-diabetic, antiviral, antibacte-rial, anti-inflammator , and anti-allergic, as well as immunomodulatory and radio-protective properties. However, most of these studies have only been conducted at laboratory-scale and mainly use water or organic solvent extraction systems. These conventional techniques have several drawbacks such as low selectivity, low extraction efficien y, solvent residue, and environmental pollution. Therefore, it is necessary to develop innovative technologies for extraction, separation, and purificatio of bioactive compounds from marine algae, including

supercritical flui extraction, subcritical water extraction, pressurized flui extraction, enzyme-assisted extraction, and membrane separation system. Further optimization and scale-up of these new technologies is crucial for successful commercial developments. Reliable and high-throughput analytical techniques need to be developed for the qualitative and quantitative analysis of these compounds in different matrices. The claimed physiological effects of some seaweed extracts or purifie compounds have, so far, only been tested in relatively fast and simple *in vitro* screening trials. Well-designed *in vivo*, animal, and human clinical studies should be carried out to systematically evaluate the health benefit and potential risks of these bioactive ingredients. Studies on the impact of processing conditions on the stability and bioavailability of these compounds are scarce. The adverse interaction or complexation of seaweed extracts and purifie compounds with other food ingredients (proteins, carbohydrates, and lipids) as well as the possible formation of toxic, allergenic, or carcinogenic substances should also be addressed.

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43 Seafood enzymes and their potential industrial application

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43.1 Introduction

The world market for enzymes grows by 7.6% per year and will reach $6 billions by 2011, as predicted in the Freedonia Group Inc. business report in 2007 [1]. Industrial enzymes are a business worth US$ 2 billion, 50% of which is contributed by food enzymes [2]. There is a steady increase in the number and applications, as well as annual turnover from food enzymes in the recent past. As at the beginning of 2001, the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) listed about 160 enzymes. Food enzymes as aids in food processing, including those from marine sources, have captured the interests of both regulators and food processors in most of the industrialized countries. In general, food enzymes are used to achieve desired properties in foods because they are more specific effective at low concentrations, active under mild conditions of pH and temperature, and are easy to inactivate after the desired transformations.

The diverse and extreme conditions in the marine environment results in a large pool of species adapted to a variety of habitat conditions. The availability of enzymes with diversifie activities in the marine environment makes seafoods and/or their by-products potential sources of enzyme recovery. Seafood processing is an export oriented organized sector across the globe, as compared to freshwater fis processing. The present status of disposition of global fis production indicates that more than 76% of fis produced goes for human consumption, while the rest goes for reduction to fis meal/oil or miscellaneous purposes [3]. These discards in the form of fis processing waste also include a large pool of bioactive molecules including enzymes. As per one estimate, worldwide processing of fis and fis products results in an annual discard of 63.6 million metric tonnes, accounting for between 40 and 45% of global fis production [4]. Enzymes recovered from seafood by-products could also help the environmental and ethical concerns surrounding discards and improve the financia status of the seafood companies.

Marine enzymes have application in the food industry, since they may be unique protein molecules not found in any terrestrial organism, or may be known enzymes from the terrestrial sources but with novel properties. Their characteristics differ from homologous proteases from warm-blooded animals such as tolerance to high salt concentration, low or high temperature, high pressure, and low nutrient availability. These characteristics of marine

enzymes are due to the prevalent conditions in their habitats, such as hydrothermal vents and oceanic waves [5].

As seafoods and/or their processing wastes can serve as one of the economically viable sources of enzymes, an attempt is made to review the types and potential industrial applications of enzymes available in seafoods and their by-products. For the purpose of this chapter, we have classifie seafood enzymes into broad categories of:

a) protein-degrading enzymes;
b) lipid-degrading enzymes;
c) carbohydrate-degrading enzymes; and
d) miscellaneous enzymes.

As seafood proteases (protein-degrading enzymes) have extensively been reviewed recently [6–9]. we have limited the section on proteases. This chapter is aimed to serve as a reference for researchers working on processing of seafoods as well as waste management.

43.2   Types of seafood enzymes and their applications

43.2.1   Protein-degrading enzymes

Protein-degrading enzymes (EC 3.4) hydrolyse peptide bonds that link amino acids together in the polypeptide chain, which form the backbone of protein molecules. Proteases are characterized as either exopeptidases or endopeptidases. Endopeptidases contribute up to 48% of all industrial enzymes [6] and are more important than exopeptidases [10]. Proteases can be classifie on the basis of their optimal pH (acid, neutral, and basic), their similarities to well characterized proteases (trypsin-like, chymotrypsin-like, chymosin-like, and cathepsin-like, etc.), on the basis of substrate specificity or on the basis of their mode of catalysis (serine, cysteine, aspartyl, and metalloproteases). As mentioned earlier, for obvious reasons, we have not reviewed the types and properties of seafood proteases. Instead, major proteases occurring in different seafoods and/or their body parts/by-products along with their typical characteristics and sources are listed in Table 43.1. The optimal pH and temperature of proteases obtained from different body portions varies within and between different fis species, as shown in Table 43.2. Fish viscera are a rich source of proteases such as trypsin, trypsin-like enzymes, chymotrypsin, collagenase, elastase, carboxylpeptidase, and carboxylesterase, while fis muscle contains catheptic and other hydrolytic enzymes [5,11]. In addition, seafood proteases, apart from being species dependent, also indicate seasonal variations in their activity as well as quantity [12]. The characteristic properties of seafood enzymes include higher catalytic efficien y at low temperatures, cold stability, lower sensitivity to substrate concentrations, and greater stability at broader pH range [5]. Protease, particularly extracellular proteases are likely to play a pivotal role in making nutrients available in cold environments [12,13].

43.2.1.1   *Applications of proteases*

The possible potential applications of seafood protease and other enzymes are summarized in Fig. 43.1. Seafood proteases are also used as processing aids in many products including baked goods, fermented beverages, milk, and dairy foods such as cheese, eggs, and their

Table 43.1 Enzyme code, occurrence, molecular weight, and optimum activity of different types of proteases

Proteases	Enzyme code	Body parts	Molecular Weight (kDa)	Optimum activity	Reference
Trypsins	EC 3.4.21.4	Pyloric caeca, pancreatic tissue or intestine	22.5–24	pH 7.5–10, 35–45°C	[5]
Chymotrypsins	EC 3.4.21.1	Pyloric caeca	25–28	pH 9	[5]
Elastase	EC 3.4.21.36	Pancreas, intestine	24.8–28	pH 5–9, 45°C	[5]
Pepsin	EC. 3.4.23.1	Digestive glands, stomach tissue	27–42	pH 2–4, 37–55°C	[5]
Chymosin	EC. 3.4.23.4	Gastric mucosa	33.8	pH 2.2–3.5	[5,9]
Gastricin	EC. 3.4.23.3	Gastric juices	32.3 and 33.9	pH 3	[5]
Cathepsin#*	1(B), 15(L), 16(H), 24(T), 27(S), 38(K), 41(F), 42(O), 43(V)	Muscle tissue, lysosomes, sarcoplasm, & extracellular matrix of connective tissue	13.6–39.5	pH 3.5–8	[5,13,26]
Cysteine					
Aspartate	EC 3.4.23.5 (D)*				
Serine	EC 3.4.16.5 (A)*				
Collagenase	EC 3.4.21.11	Digestive glands	25–125	pH 6.5–8, 55°C	[5,26]
Calpains	EC 3.4.22.17	Skeletal muscle	108	pH 7.5, 30°C	[6,69]

#Stands for EC3.4.22.

The alphabet enclosed in the bracket signifies the type of cathepsin. E.g. 1(B) signifies Cathepsin B with EC 3.4.22.1.

Table 43.2 Types of proteases, their characteristic features, and source

Type of protease	Sub classes	Characteristic feature	Sources	Reference
Serine protease	Trypsin and trypsin-like enzymes	Cleaves ingested proteins, activate precursor form of other proteases, and hydrolyses native globular protein	Anchovy, Greenland cod, Atlantic cod, capelin, mullet, sardine, catfish, starfish, crayfish, cunner' Atlantic salmon, & krill	[8,26]
	Chymotrypsin	Hydrolyses various protein substrate (casein, collagen, & bovine serum albumin) known to clot milk	Anchovy, Atlantic cod, capelin, herring, rainbow trout, & spiny dogfish	[8,26]
	Elastase	Ability to digest the elastic, fibrous protein of connective tissue – elastin	Atlantic cod, bluefin tuna, catfish, dover sole, eel, sea bass, monk fish, yellow tail, and teleost tuna	[5,8]
	Collagenase (intestinal)	Autolysis of crustacean muscle tissue during post harvest storage and hydrolyses the triple type I, II, & III tropocollagen molecules	Fiddler crab, cray fish, king crab, & Atlantic cod	[8]
Aspartate protease	Pepsin	Irriversible inactivation at alkaline conditions	Atlantic cod, capelin, Greenland cod, Polar cod, sardine, American smelt, dogfish, & Monterey sardine	[5,7]
	Chymosin	High milk clotting to proteolytic ratio, inability to inactivate ribonuclease, & instability in 6M urea	Harp seals	[8]
	Gastricsin	Acidic protease resembles pepsin, but differs in catalytic properties	Atalantic cod, salmon, & hake	[5,8]
Cysteine/thiol protease	Calpain	Ca^{2+} activated protease involved in post-mortem ageing process and promotes flesh softening	Sea bass	[69]
	Cathepsin	Lysosomal protease involved in texture softening of fish flesh	Horse clam, mussel, & surf clam	[5]
Metalloprotease	Collagenase (muscle)	Partial degradation of collagen and other extracellular matrix proteins	Crab, lobster, prawn, rockfish, & Atlantic cod	[26]
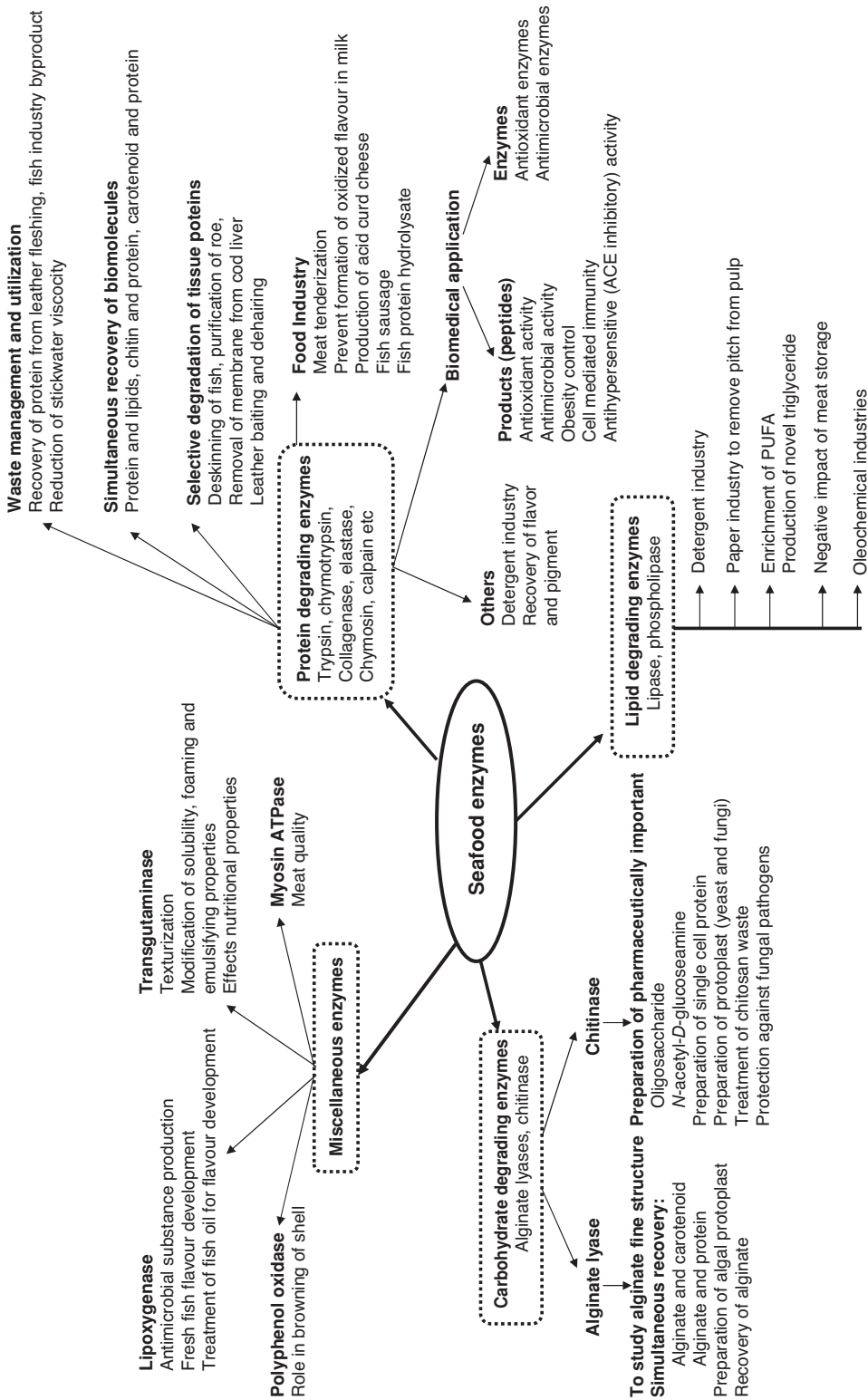
Fig. 43.1 Potential and scope for the application of seafood enzymes in various industries.

products, and meat and their products for the production of protein hydrolysate and fl vour compounds [14]. Digestive proteases from marine invertebrates fin applications in various industries such as the food industry, leather industry, and detergent industry, etc. Digestive proteolytic enzymes from cold-adapted aquatic organisms possess unique properties compared to mammalian proteases [15]. The subject of recovering enzymes from seafoods and/or their waste has been extensively reviewed by An and Visessanguan [16]. Protease extracted from endogenous sources has been applied to produce fis protein hydrolysate in both capelin and seal meat [17,18]. Proteases from marine sponges and crabs are known to degrade casein and hide powders [13]. Protein hydrolysates prepared from seafoods and/or their by-products are reported to exhibit various biological functionalities, including antioxidant, anticancer, antihypersensitive, and antidiabetic properties [19].

Proteases from seafoods can offer a better tool for selectively breaking the tissue that connects the soft tissue to muscle and gonads, where it is difficul to remove the skin by mechanical means [20]. Similarly, fis pepsin is reported to hydrolyse the supportive tissue that envelops salmon, and trout roe and roe sack [6]. Seafood enzymes can also be used for collagen extraction and it has been reported that pepsin in combination with acid extraction increases the yield of collagen [21]. Certain fis enzymes are excellent catalysts at low temperature, which is advantageous in some food processing operations [5,7]. Recently, the use of alkaline proteinases from marine digestive organs, especially trypsin, has increased remarkably, as they are stable and active under high temperature and pH, and presence of surfactants/oxidizing agents [9]. Endogenous proteases present in seafoods or their wastes can fin potential application in simultaneous recovery of lipids and proteins from fis [22], and carotenoids, chitin, and proteins from shrimp [23,24] and crab [6] wastes. They can also be used in extraction of fl vour compounds from shells and other materials [6,25], debittering of hydrolysate, viscosity reduction of fis meal stick-water during drying and removal of the collageneous membrane surrounding cod liver prior to canning and tenderization of meat [26].

43.2.2 Lipid-degrading enzymes

Lipids are one of the major parts of the Earth's biomass and lipid-degrading enzymes play an important role in the turnover of these water insoluble compounds. For the purpose of this review, these enzymes have been classifie under the two headings of lipases and phospholipases.

43.2.2.1 *Lipases*

Lipases (EC 3.1.1.1) are enzymes found widely in nature, including seafoods and have considerable physiological significanc and industrial applications. Lipases are known to catalyze reactions such as esterification hydrolysis, or exchange of fatty acids in esters [27]. The diversity and effectiveness of lipases from seafoods can be attributed to factors such as food adaptation, migrations, seasonal variations, etc. Seafood lipases also have an important physiological role in hydrolysis of water insoluble triacylglycerols (TAG) to more polar diacylglycerol (DAG), monoacylglycerol (MAG), free fatty acids (FFA), and glycerol for absorption and transport through the membrane [8]. For instance, in some carnivorous fish carbohydrates are not easily available, therefore lipids are important sources of energy [28,29].

Pancreatic lipases are necessary for absorption and digestion of lipids, including MAG, DAG, and TAG. Fatty acid digestibility in fis decreases with increase in chain length and increases with increase in degree of unsaturation [28,30]. Lipases have been characterized from various aquatic animals such as cod [28], mackerel [31], and salmon [32]. Generally, lipases are active over a wider temperature range (-20 to $60°C$), with most lipases active between 30 and $45°C$ with a few exceptions. The pH optima of most of the lipases lie between 7 and 9. Lipase activity in marine fis is mainly detected in muscle, gut, and liver. Bile salt activated lipases (BAL), which hydrolyze cholesterol/vitamin esters, are one of the predominant lipases present in the pancreas of marine fis [8].

43.2.2.2   *Phospholipases (PL)*

PL are lipolytic enzymes that hydrolyze phospholipids and are grouped into two catogeries, acylhydrolases and phosphodiesterase. Unlike classic esterase, their natural substrate is insoluble in water and their activity is at maximum only when the enzyme is adsorbed onto a lipid water interface. The most studied types of phospholipids are PLA_1, PLA_2, and PLC [33]. PLA_1 plays an important role in wax ester synthesis [34], PLA_2 plays a role in wax ester synthesis using carbohydrate and amino acids as precursors, and PLC plays an important role in signal transduction and provides potential secondary messengers [35]. PLA_2 hydrolyzes essential dietary phospholipids in marine teleosts, regulates membrane lipids modificatio as a response to environmental changes, and provides fatty acids as substrates for metabolic energy and biosynthesis of prostaglandins. Generally PLA_2 is Ca^{2+} dependent and has an optimum pH in alkaline condition and is stable against acid and heat treatment [36]. PLA_2 from hepatopancreas (Isoform DE-1 and DE-2) and calcium dependent PLA_2 from pyloric ceacum of red sea bream (*Pagrus major*) have been purifie and characterized [37].

PL have been studied from various fis such as pollock, *Pollachius virens* [8], pyloric caeca [36] and hepatopancreas of red sea bream [37], liver of trout [38], and cod muscle [8]. The optimum temperature and pH ranges of these PL are reported to be 30 to $45°C$ and 8 to 10, respectively. However, lysosomal PL of cod (pH 4) and PLA_1 of Bonito (pH 6.5–7; temperature 20–$30°C$) are exceptions to this. Furthermore, unlike porcine lipase, BAL (a carboxyl ester lipase) purifie and characterized recently from hepatopancreas of red sea bream (*Pagrus major*) is reported to efficientl hydrolyze ethyl esters of polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) and eicosapentaenoic acid (EPA) [29].

43.2.2.3   *Applications of lipases and their role in seafood quality*

Lipolytic reactions play an important role in post-harvest quality deterioration of chilled and/or frozen seafood as well as fis oils. FFA formed on hydrolysis may promote lipid oxidation and may also be responsible for off-fl vours or off-odours, the destruction of some vitamins and amino acids, the changes in texture, and water holding capacity of muscle proteins [39]. During storage under frozen condition, lipase activity in oils from sardine and ribbon fis decreased after 60 days and reached the least activity after 180 days of storage [40].

Lipases have applications in dairy, detergents, oleochemical, paper, and food industries, apart from use in the production of biofuels. In oleochemical industries, lipase application saves energy and minimizes thermal degradation during alcoholysis, hydrolysis, and glycerolysis [41]. In recent years, marine oils have received much attention due to their positive health effects attributed to omega-3 (n-3 or ω-3) PUFA, namely EPA and docosahexaenoic

acid (DHA) [42]. Native enzymes from seafoods or their by-products are better options for enriching PUFA in fis oil [14]

43.2.3 Carbohydrate-degrading enzymes

Marine organisms feed on seaweed and produce a mixture of carbohydrate-degrading enzymes. In this section, we are restricted to occurrence of alginate lyases and chitinase occurring in seafoods and/or their by-products, and not from marine microbial sources. The sources and characteristics of alginate lyases and chitinase in seafoods are listed in Table 43.3.

43.2.3.1 *Alginate lyases*

Alginate lyases are important carbohydrate-degrading enzymes that allow marine organisms to efficientl harness all of the energy available. Alginate is a copolymer of alpha-*L*-guluronate (G) and its C5 epimer beta-*D*-mannuronate (M) arranged as homopolymeric G block, M block, alternating GM, or random heteropolymeric. Alginate lyases have been characterized based on their dominant cleaving action on M-rich or G-rich alginates as poly (M) lyases and Poly (G) lyases. They have been isolated from many sources, including marine algae, marine molluscs, and a wide range of micro-organisms. Although most marine organisms produce single alginate lyase with define substrate specificit , some produce two or more types. In marine molluscs, lyases has been isolated from gut, gland, style, or hepatopancreas [43]

43.2.3.2 *Chitinase*

Chitinase are endo-β-*N*-acetylglucoseaminidases, which randomly hydrolyze β-1, 4-linkage in poly- and oligosaccharide of *N*-acetylglucoseamine. The dimer of β-1, 4 linked *N*- acetyl-glucoseamine (chitobiose) is the main product of chitinase activity. Furthermore, these dimers are hydrolyzed by chitobiase (Exo-*N* acetyl β-*D*-glucoseaminidase or NAGase) to give single units of *N*-acetylglucoseamine [44]. In marine organism, chitinase and chitobiase are associated with the moulting process of insects and crustaceans. Chitinase has been characterized from digestive track of various aquatic fis such as cod [45], livers of a prawn [46] and squid [47], and stomach of Japanese eel [48].

43.2.3.3 *Applications of carbohydrate-degrading enzymes*

Alginate lyases from marine source have been used for evaluating algal cell wall development [49] and for studying structure of alginate molecules to establish as to how chemical composition influence their physical properties [50]. The structural studies are important to assess alginate polysaccharides, which in future can be used in combination with other chemotherapeutics.

Chitinases have application in preparation of pharmaceutically important oligosaccharides such as *N*-acetyl-*D*-glucosamine and glucosamine. The leading applications of chitinases are protection against fungal pathogens, as chitin is the primary constituent of the fungal cell wall [51]. Chitosan/chitin oligosaccharides produced by chitinases, act as antioxidants by scavenging different free radicals responsible for a number of chronic diseases [51].

Table 43.3 Enzyme code, occurrence, and properties of carbohydrate degrading and miscellaneous enzymes from seafood

Enzyme	Enzyme code	Body parts	Molecular weight (kDa)	Optimum activity	Reference
Carbohydrate degrading enzyme					
Alginate lyase	EC 4.2.2.3	Mid-gut gland and hepatopancreas	32–74	pH 5.6–9.6, 30–37°C	[50]
	EC 4.2.2.11				
Mannuronate lyases					
Guluronate lyases					
Chitinase	EC 3.2.1.14	Liver, stomach, and intestine	25–120	pH 4.5–7	[8]
Chitobiase	EC 3.2.1.30				
Miscellaneous enzymes					
Myosin ATPase	EC 3.6.1.3	Muscle tissue	480	pH 9	[55]
Polyphenol oxidase	EC 1.10.3.1	Shells	30–210	pH 6–8, 30–45°C	[70]
Lipoxygenase	EC1.13.11.12	Skin, gills, eggs, and hemolymph	75–104	pH 6.5, 38°C	[52]
Transglutaminases	EC 2.3.2.13	Muscle and liver	84–95	pH 8, 20–40°C	[8,26]

43.2.4 **Miscellaneous enzymes**

43.2.4.1 *Lipoxygenase (LOX)*

LOX is a dioxygenase that catalyzes the oxygenation of PUFA containing a cis, cis-1, 4-pentadiene system to hydroperoxides [52]. LOX activity is reported in a few marine organisms such as coral, sea urchin, grey mullet, skin and gills of trout, and eggs of starfish Although considerable amounts of LOX in the muscle or liver of various marine organisms is not reported, it was detected in large amounts in the light muscles of lake herring [53]. LOX exists in multiple forms, as 5-LOX, 12-LOX, and 15-LOX, in the tissues of shrimp and grey mullet. Of these, 12-LOX has the highest activity and is in higher concentrations (almost 20-fold) than the other two [52]. The suitable substrates for LOX are PUFA containing a series of methylene interrupted *cis* double bonds [54]. LOX from shrimp has shown increased reactivity with PUFA substrate of increased unsaturation, while those from grey mullet act in an opposite manner [52].

43.2.4.2 *Myosin ATPases*

Myosin ATPases are hexamers of two heavy chain subunits and four light chain subunits, which hydrolyze adenosine-5′-triphosphates (ATP) to adenosine-5′-diphosphates (ADP) in fis muscle [55]. In the absence of actin, myosin ATPase is activated by Ca^{2+} (3–5 mM) but inhibited by Mg^{2+}; however, when actin is present, it is activated by both Mg^{2+} and Ca^{2+}. They are also activated in the presence of K^{+} and ethylenediaminetetra acetic acid (EDTA). Myosin ATPase in fis ordinary muscle shows optimum activity at around pH 6 to 9, the highest being at pH 9 [55].

43.2.4.3 *Polyphenol oxidases (PPO)*

PPO are a mixture of phenoloxidases (monophenol oxidase and cathecol oxidase), phenolase, cathecol oxidase, cresolase, diphenol oxidase, and tyrosinase. They are generally found in shellfis and their by-products including shrimp, prawns, lobster, and cuttlefis [26]. Stability of PPO varies over a number of factors such as temperature, pH, substrate, ionic strength, buffer system, and time of incubation, apart from source and environmental factors [56]. Most of the PPO are heat-labile, except lobster PPO, which is reported to exhibit thermotolerance [57]. PPO in crustacean species results in post-harvest melanosis and related quality problems [58].

43.2.4.4 *Transglutaminase (TG)*

TG (EC 2.3.2.13) is an endogenous fis enzyme that catalyzes acyl transfer reaction between γ-carboxamide groups of glutamine residues in proteins, polypeptides, and a variety of primary amines [26]. TG is a sulphydryl enzyme with a conserved pentapeptide active site sequence (Tyr-Gly-Gln-Cys-Trp) [59] and TG from seafood species are generally monomeric proteins. TG activity has been reported in the muscles of rainbow trout, red sea bream, atka mackerel [60,61], scallop, botan shrimp, squid [62], and walleye pollock liver [63]. Post-harvest TG activity rapidly decreases in fis and is completely inactivated by freezing.

43.2.4.5 *Applications of miscellaneous enzymes*

LOX plays an important role in oocyte maturation, regulation of membrane permeability, antimicrobial substance production and formation of typical seaweed, and fresh fish flavour [52]. Characteristic aromas for freshly harvested fish are derived from reaction of PUFA catalyzed by the endogenous LOX yielding volatile alcohols and aldehydes [64]. Since LOX can be induced by stress, different isoenzymes of LOX can be formed under different environmental conditions [65], which in turn can result in various type of flavour (undesirable or desirable) in post-harvest scenario.

As mentioned earlier, apart from physiological importance to crustaceans, PPO play an important role in post-harvest seafood quality [26]. Similarly, TG has industrial applications because of its effect on functionability of proteins [66], including cross-linkage of proteins during surimi production [67]. The effect of cross-linking is useful in modification of solubility, foaming, whipping, and emulsifying properties of proteins as well as improving nutritional properties by protecting lysine and other essential amino acids from undesirable reactions [68]. Myosin ATPase has a role in meat quality changes [55].

43.3   Conclusions

Adaptability to extreme conditions makes seafood enzymes superior to their terrestrial counterparts. Fish processing by-products contain several enzymes. As these by-products are available in large quantities (>50% of live weight), they can serve as potential sources of marine enzymes. Among by-products of marine fish and shellfish viscera are the major source of seafood enzymes. For effective application of seafood enzymes by the food industry, research is required to evaluate these enzymes as novel tools in conjunction with conventional techniques. For instance, marine enzymes can be used in processes to recover biomolecules such as carotenoid, PUFA rich oil, and chitin from various seafoods and/or their by-products. Furthermore, upon establishment of characteristics and applications, marine enzymes can be produced on a large scale at an affordable cost using novel molecular and/or biological techniques. Thus, seafood enzymes have a myriad of potential applications. Increased interest is evinced by research attempting to develop processes/methods to integrate utilization of fish industry wastes with fish processing. This would enhance the plant economy and decreasing the pollution/environmental problems associated with their disposal. It would also result in value added products such as enzymes and pigments from marine resources.

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