Biochemical Analyses in Seafood and Seafood Products – Basic Concepts

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1.1 World Catch and Harvest

Seafood has by far the greatest variety of all animal-based foods. Whereas the species consumed as warm-blooded mammals (beef, pork, lamb, goat, and donkey) or poultry (hen, turkey, geese, and duck) are represented by very few species. The fish group alone is represented by 25,000–35,000 species. However, only a little proportion of this large number of about 5% is present in the world's oceans in amounts huge enough to allow an economical use (catch and following processing). Further, only some of these 5% have the desired sensory properties and give a good or satisfying fillet yield that catching and processing them can be justified.

Another difference compared with land-living animals is the fact that the quality (size, state of maturity, nutritional status, infestation with parasites, burden of pollutants, etc.) of aquatic animals when captured by fishing techniques is with few exceptions completely unknown. Although land-based animals are today tailor made according to industries and consumers wishes in weight, body composition, appearance, and sensory properties, in the case of captured seafood we have to accept what we find in the fishing net despite modern advanced technologies.

Further, fish and other seafood are highly perishable products when stored without chilling. They deteriorate at ambient temperature in a few days, and only correct storage of wet fish in melting ice or of certain products at chilled temperatures can prolong the shelf life up to weeks or months.

The total world seafood supply is amounted to 143 million tons. The world's aquaculture provided 52 million tons (36%), and the captured fish, 91 million tons (64%) of the total supply. Although the amount of captured fish is almost constant at a level around 90 million tons/ year since 1990 after a continuous growth for more than 40 years, aquaculture is dramatically growing (1960: 2 million tons, 1970: 4 million tons, 1980: 7 million tons, 1990: 16 million tons, 2000: 40 million tons, including plants). The stagnation of captured fish is mainly due to fully exploited or partially overfished stocks.

The most important primary product producing countries of marine and inland (freshwater) fisheries in 2005 were China (17.1 million tons), Peru (9.4 million tons), the United States (4.9 million tons), Indonesia (4.4 million tons), Chile (4.3 million tons), Japan

(4.1 million tons), India (3.5 million tons), Russia (3.2 million tons), Thailand (2.6 million tons), and Norway (2.4 million tons). The top 10 species being caught in huge amounts in 2005, were Anchoveta (10.2 million tons), Alaska Pollock (2.8 million tons), Atlantic herring (2.3 million tons), Skipjack tuna (2.3 million tons), Blue whiting (2.1 million tons), Chub mackerel (2.0 million tons), Chilean jack mackerel (1.7 million tons), Japanese anchovy (1.6 million tons), Largehead hairtail (1.4 million tons), and Yellowfin tuna (1.3 million tons). The major aquaculture (excluding plants) producers (>1 million tons) in 2005 were China (32.4 million tons, whereof the major part are cyprinids like carp), India (2.8 million tons), Vietnam (1.4 million tons, mostly Pangasius species), Indonesia (1.1 million tons), and Thailand (1.1 million tons).

By major groupings, fish is the top group in aquaculture at 47.4% by quantity. Aquatic plants that are popular in Southeast Asia are second in quantity at 23.4%, whereas crustaceans are fourth by quantity at 6.2% but second by value at 20.4%. Mollusks (bivalves and cephalopods) are the third most important group both by quantity and by value at 22.3% and 14.2%, respectively. About 75% of the world's total seafood supply is used for human consumption, 25% is converted into fishmeal and other non-food products, 40% is consumed as wet fish without any further technological processing or preservation, about 20% is converted into deep frozen products, 8% is transformed into cured products, and another 8% into canned products.

1.2 Variability of Aquatic Animals

The variability of aquatic animals can be described and explained in many different ways. Based on taxonomic criteria, we have different groups such as bony and cartilaginous fishes, crustaceans, and molluscs, which are very different from each other in appearance, composition, and nutritive properties. When concentrating on fish as the major group contributing to the world's fish supply, we arrange them in order according to their shape into round fish, flat fishes, eel like fishes, and so forth, or according to their occurrence in the ocean's water column into pelagic fish, bottom fish, demersal fish, and ground fish. We can also group them according to their fat content into three groups: lean fish species (<1% fat), medium fatty fish species (>1% to <10% fat), and fatty fish species (>10% fat).

However, these are all very rough classifications. In addition, the main difficulty in the analysis of fish and other seafood is that there is not only a big variation between groups of species and species but also within a given species. Not only weight and length are varying with age but also other factors such as proximate composition, mineral, and trace element content, which are subject to variations based on state of maturity, fishing area, season, pollution of water, and so on. This means that each fish can be different and unique, and before analysing fish, a careful consideration has to be made if the variation is important and if it is worth or essential knowing (leading to analysis of individuals) or if a more general impression about the target component is sufficient (pooled samples).

1.3 Special Problems with Aquatic Animals

The main problem with aquatic animals is the fact that from the moment that they are caught or harvested, a change in properties starts, which continues until a state of spoilage is reached. After catch and harvest, not only spoilage and freshness parameters are changing due to metabolic (autolytic) and microbiological processes but also the microbial flora is changing. Besides this more general aspect, some groups offer special problems to which a lot of attention has to be given: aquatic animals may contain parasites (e.g., nematodes, cestodes) that can be harmful to humans when they enter live and intact into the human body.

Predatory fish species such as sharks, which are at the end of the marine food web, can accumulate mercury during their long life span to quantities that exceed legal limits. Toxins from dinoflagellates can accumulate in bivalve mollusks, leading to several diseases such as diarrhetic shellfish poisoning (DSP), paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), and amnesic shellfish poisoning (ASP), and in fish, leading to ciguatera or maitotoxin poisoning. In the digestive glands of molluscs (hepatopancreas) such as cephalopods and mussels, cadmium is accumulated to amounts that exceed any legal limits by far. When not eviscerated immediately after catch, cadmium from hepatopancreas penetrates into the edible part (mantle) during storage, leading to elevated cadmium concentrations also in this body compartment. In products that have not undergone thermal treatment and that are offered to the consumer as ready to eat (e.g., cold smoked products, gravad products, sushi, and sashimi), there is an inherent microbial risk.

Fish and other aquatic animals from areas that are polluted (rivers, inshore waters, estuaries, seas with no or limited water exchange with world oceans such as Baltic Sea, Mediterranean Sea, Caspian Sea, or Black Sea) can carry a high burden of environmental pollutants, especially in their organs responsible for detoxification such as liver and kidney. Aquatic animals from some areas of the world can carry viruses and microorganisms (e.g., *Vibrio* sp.) that are harmful to human health and must be destroyed or removed before marketing of the products.

1.4 Biochemical Composition of Fish

Biochemical constituents of any fish species denote its nutritional and energy status. The biochemical composition varies from species to species, within the same species and within the same individual. Knowledge on the biochemical changes of the fish is essential for understanding the metabolism of different populations, for providing an estimation of the energy content and for understanding the biochemical circulation of elements. It is quite evident that the frequency of change in the composition of biochemical constituents of biota varies not only with the environment changes but also with seasons. In tropical countries much is known of chemical composition of marine fishes, but little of freshwater fishes except for some stray records on hill stream fishes.

Malnutrition is the major problem of the third world countries and India is no exception to it. Many scientists, not only in India but all over the world, are trying to find out the ways and means to combat this problem. Protein deficiency leads to various clinical and sub-clinical syndromes, such as impaired health, lowered resistance to infection and susceptibility to disease. In the developed countries related health problems arise from over composition of fat, saturated fatty acids, cholesterol, etc. Various human health organizations in the world have emphasized the importance of fish as a vital food, to meet the nutritional requirements for achieving ideal body weights. Fish is a rich source of nutrients with relatively low cholesterol content and high content of easily digestible quality protein. The most important constituents of fish muscle are protein, which generally varies from 17 to 25% in fresh condition. Fish is generally less expensive in comparison to other protein foods. Historically, the Oceans were considered limitless and thought to harbor more than enough fish to feed an ever-increasing human population. However, the demands of a growing population, particularly in poorer countries, now far outstrip the 112 sustainable yields of the seas. Fish are an important element of the human food supply, and fishing is an important factor in global employment

Proper knowledge on the biochemical composition of fish finds application in several areas. Today there is an ever-increasing awareness about healthy food and fish is finding more acceptance because of its special nutritional qualities. In this context a proper understanding about the biochemical constituents of fish has become a primary requirement for the nutritionists and dieticians. Fish and fishery products are used in animal feeds. In this case also, proper data on the biochemical composition is essential for formulating such products.

Another vital area where accurate information on biochemical composition is a must is processing and preservation of fish and fishery products. Fish is an easily perishable commodity and deterioration in quality is due to the changes taking place to the various constituents like proteins, lipids etc. Information on the biochemical constituents will help a processing technologist to define the optimum processing and storage conditions, so that the quality is preserved to the maximum extent.

1.5 Benefits and Risks

Seafood is a rich source for a great number of nutritive and important components. The high amount of long-chain polyunsaturated fatty acids of the n-3 series such as eicosapentanoic acid (20:5) and docosahexanoic acid (22:6); the vitamins A, D, E, and B_{12}; the well-balanced content of essential amino acids; the high amount of taurine; the presence of antioxidants such as tocopherols; the exceptional concentrations of essential elements such as selenium and iodine; and the good digestibility of fish protein due to low amounts of connective tissue are some examples of the many benefits seafood offers, when consumed.

On the other hand, we have the risk of viruses and microorganisms; we are confronted with toxins in mussels and fish; we have sometimes a parasitical problem; we may find high

amounts of inorganic toxic elements and organic pollutants (POP, persistent organic pollutants), and residues of pharmaceuticals and hormones used in aquaculture can be detected and more. Considering the great variability of seafood described here, a tremendous amount of analytic work in seafood has to be done.

1.6 Trends and Outlook

In the future, most chemical and biochemical analytical methods that use a huge amount of chemicals and manpower will be substituted by instrumental methods that are more reliable, more cost efficient, and more environmental friendly. Some methods that are well known such as k-value or TVB-N will disappear. Analytical instruments that are simple to use, robust, and have a wide range of applicability will be built. This next generation of instruments will then also find its way into the fish industry and fish inspection. Although the lipids in seafood are analyzed very intensively, the protein and peptides are analyzed to a much lesser extent. Recent findings show that seafood contains important functional proteins and peptides. More research and development of analytical methodology will be initiated by these new findings.

Almost all analytical methods for seafood analysis will be developed further to avoid time and chemicals and to minimize sample preparation and digestion steps. The method of the future will analyze a well-homogenized sample without any other sample preparatory steps except homogenizing. However, all progress in analytical methods and instrumentation needs an analyst who is responsible and follows the guidelines and advice for analytical quality assurance.

2 Proximate Composition of Fish

2.1 Introduction

The proximate composition in most fish and shellfish is primarily water, proteins, and lipids. In fish meat these constituents make up about 98% of the total mass, and the other minor constituents include carbohydrates, vitamins, and minerals. Proximate data on different fish species are collected in databases such as the Fish Base (www.fishbase.org); however, the chemical composition of fish generally varies due to seasons, geographical locations, stages of maturity, and sizes, and so on. Therefore, to ensure obtaining data on the exact proximate composition, analysis should be performed on the specific samples.

2.2 Water content

Water is the most abundant and surely the most frequently overlooked component in foods. It is estimated that over 35% of our total water intake comes from the moisture in the foods we consume.

The control of water activity in foods is an important tool for extending shelf life. It is responsible for the quality of foods affected by microbiological, chemical, and physical changes. The physical properties, quantity, and quality of water within the food have a strong impact on food effectiveness, quality attributes, shelf life, textural properties, and processing. Food-preservation processes have a common goal of extending the shelf life of foods to allow for storage and convenient distribution. The activity of microorganisms is the first and most dangerous limitation of shelf life. Water is essential for microorganisms that may cause food spoilage if they are present in a food that offers them favourable conditions for growth. Hence, many food preservation techniques were developed to reduce the availability or activity of water in order to eliminate the danger of microbial spoilage.

Water content in fish can be determined by simple drying methods. Using conventional air ovens, a common practice has been to dry the sample at 105°C for 12 h, which by experience has shown satisfactory drying of fish and fish products. To ensure complete drying, the sample can be dried to constant weight. Other methods refer to 101°C for 24 h by conventional ovens and 70°C for 24 h using vacuum ovens. The sample is weighed in a container, and after heating the sample is cooled and weighed again.

2.3 Crude Protein

Protein analysis is highly important for the food industry, including the fish industry. Both the content and the properties of the proteins are important for the value and the quality of the products. Both for quality control and food labelling it is therefore important to have methods to determine not only the total content of proteins in a raw material or a product, but it is also important to have methods to determine the type and the origin of the proteins present. For product and process development it is important to have methods to determine the properties of the proteins and how these change during processing and storage, and how these properties are influenced by food additives and other components.

Fish provides about 14% of the world's need for animal proteins and 4%–5% of the total protein requirement. Both the amino acid composition and the digestibility of fish proteins are excellent. Fish are regarded as an excellent source of high-quality protein, particularly the essential amino acids lysine and methionine. In addition to the high nutritional value, fish proteins also have good functional properties such as water-holding capacity, gelling, emulsification, and textural properties.

The total content of proteins is usually determined by the Kjeldahl or the Dumas method. It is also possible to determine the nitrogen content using elemental analysis (C/N analyzers). It is important that the methods to analyze food proteins are robust. This means that it should be possible to use the method on different types of foods, both different types of raw materials and processed foods, and that other components in the food such as lipids and pigments should not interfere with the analysis. The method should also require minimal

sample pre-treatment to decrease analytical error and reduce costs. The Kjeldahl method was first published in 1883 but has been extensively modified since then. The method includes sample digestion, neutralization, distillation, and trapping of ammonia and titration steps. The advantage of this method is that it gives accurate results for all types of samples. This method is used as a reference method by many national and international organizations.

2.4 Lipids

Lipids include a wide heterogeneous group of compounds. Lipids are defined as the fraction of any biological material extractable by solvents of low polarity. As can be seen, the definition itself is not a precise one, but that is thought to be the best to include all compounds belonging to this group. Any material extracted with 'fat solvents' like ethyl alcohol, ether, chloroform, hexane, petroleum ether etc. is classified as a lipid. The important type of compounds included in this group are fatty acids, glycerides, phosphoglycerides, sphingolipids, aliphatic alcohols and waxes, steroids and combination of the above type of compounds with proteins, peptides carbohydrates etc. In the case of fish tissues, the major components of lipids are triacylglycerol and phosphoglycerides, both containing long chain fatty acids. Smaller proportions of other components are also present.

Phospholipids, another important constituent of lipids are essential components of cell membranes. It is the lipid-globular protein mosaic structure that determines important functions like permeability of cell membranes, transport of various substances into and outside the cell. Various types of phospholipids are essential for the proper functioning of the cell. Unlike in the case of depot fat, the proportions of phospholipids do not show wide variation. Normally it is in the range of 0.5 to 1% of tissue.

The major chemical entity in most lipid molecules like glycerides, phospholipids, and wax esters is fatty acid. The nature of the fatty acids present in fish lipids is very complex. Fatty acids with carbon chain varying from 10 to 22 and unsaturation varying from 0-6 double bonds are of common occurrence.

Traditional methods for determination of total lipids are generally based on solvent extraction followed by gravimetric determination. The lipid yield obtained is highly dependent on the solvent system, and using a combination of polar and nonpolar solvents it is possible to extract the total lipids and not only the free lipids such as triacylglycerols. Differences in lipid yield among the methods are claimed to correlate with the extraction efficiency of the more tightly bounded polar lipids such as phospholipids

2.5 Carbohydrate

Carbohydrates are most abundant class of organic compounds found in living organisms. Carbohydrates are a major source of metabolic energy, both for plants and animals.

A diet that does not contain carbohydrate can lead to muscle breakdown, ketosis and dehydration. Carbohydrates are often classified into three broad groups: sugars (mono- and disaccharides), oligosaccharides (three to nine monosaccharides) and, polysaccharides (more than nine). The content of carbohydrates in fish muscle is low and is further influenced by conditions experienced before and during capture, which may lead to depletion of glycogen stores and thereby a decrease in the carbohydrate level. Under anoxic conditions postmortem, glycogen will continue to be metabolized, resulting in increased lactic acid along with reduced pH and eventually a gradual loss of the sweet, meaty character of fresh fish.

Some marine invertebrates on the other hand are characterized by a high content of carbohydrates; up to 10.2% and 12.5% total sugars can be found in subcuticular tissue of spiny lobster and blue crab, respectively, with the highest amounts of glucose followed by galactose and mannose. Glycogen stores of scallops are highly dependent on season (temperature, food availability, and lifecycle), and highest levels are usually reached after the summer period, showing levels up to 23%–25% glycogen of dry weight of adductor muscle. Seasonal variations of glycogen content in mussels (Mytilus edulis) are also high, showing values in the range 4%–37% of tissue dry weight.

Phenol sulphuric acid method is the most reliable and easiest method among the quantitative assays for carbohydrate estimation. This method is widely used to determine the total concentration of carbohydrate present in foods. The results are expressed in the terms of a single carbohydrate, usually glucose. In this method, in hot acidic medium glucose is dehydrated to hydroxy methyl furfural, this forms a yellow brown coloured product with phenol and has absorption maximum at 490nm. The sulphuric acid causes all non-reducing sugar to be converted to reducing sugar so that this method determines the total sugar present in foods. The method detects all classes of carbohydrates, including mono-, di, oligo- and polysaccharides. Although the method detects almost all carbohydrates, the absorptivity of the different carbohydrates varies. This method is non stoichiometric and so it is necessary to prepare a calibration curve using a series of standards of known concentration of carbohydrate.

2.6 Ash

The ash content is a measure of the total amount of minerals present within a food, whereas the mineral content is a measure of the amount of specific inorganic components present within a food, such as Ca, Na, K and Cl. Determination of the ash and mineral content of foods is important for a number of reasons:

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals (the analyte) can be distinguished from all the other components (the matrix) within a food in some measurable way. The most

widely used methods are based on the fact that minerals are not destroyed by heating, and that they have a low volatility compared to other food components. The three main types of analytical procedure used to determine the ash content of foods are based on this principle: dry ashing, wet ashing and low temperature plasma dry ashing. The method chosen for a particular analysis depends on the reason for carrying out the analysis, the type of food analyzed and the equipment available. Ashing may also be used as the first step in preparing samples for analysis of specific minerals, by atomic spectroscopy. Ash contents of fresh foods rarely exceed 5%, although some processed foods can have ash contents as high as 12%, e.g., dried beef.

3 Proteins and Amino acids

3.1 Proteins

3.1.1 Introduction

Proteins are among the fundamental molecules of biology. They are common to all life, are present in every cell on Earth today, and are responsible for most of the complex functions that make life possible. There are an estimated 100,000 different proteins in the human body alone. In plants, an inventory of more than 13,000 plant proteins has been cataloged. They are also the major structural constituent of living systems. According to the central dogma of molecular biology, information is transferred from DNA to RNA to proteins. DNA functions as a storage medium for the information necessary to synthesize proteins, and RNA is responsible for (among other things) the translation of this information into protein molecules.

Fish muscle proteins can be divided into three groups, sarcoplasmic, myofibrillar, and connective proteins, based on differences in solubility. The sarcoplasmic proteins consist mainly of enzymes and can be extracted using water or buffers with low ionic strength such as for instance 50 mM phosphate buffer. The myofibrillar proteins, also called the salt-soluble proteins can be extracted in buffers with an ionic strength of >0.3. The connective tissue proteins are often called the insoluble proteins and can be extracted using alkali or acid. The methods for extraction are not standardized so the amount of proteins extracted will vary with the method used. However, changes in solubility can be used to measure changes in protein structure caused by denaturation during storage and processing. Fish muscle proteins are more sensitive and less stable than proteins from mammals.

3.1.2 Analysis of Soluble Proteins

There are many indirect colorimetric methods to determine protein content, and a few of them will be discussed here. The Biuret method is based on the formation of complexes between copper salts and peptide bonds under alkaline conditions. The purple complex is relatively stable and has an absorption maximum at 540–560 nm. A standard curve is needed, but the method is simple and inexpensive. The method is not very sensitive, measuring

concentrations between 1 and 10 mg/mL. The sensitivity can be increased by measuring absorbance at 310 nm or by increasing the time for the Biuret reaction. However, some of these methods reduce the speed and simplicity of the method. The Lowry method is based on a Biuret-type reaction between protein and copper (II) ions under alkaline conditions, the complexes react with the Folin-phenol reagent a mixture of phosphotungstic acid and phosphomolybdic acid in phenol. The product becomes reduced to molybdenum/tungsten blue and can be measured at 750 nm. The reactions are highly pH dependent. Peterson have reviewed the Lowry method and listed interfering substances, giving upper tolerable limits for a long range of these as well as some methods for coping with the effect of these substances. Reducing agents and sucrose as well as several common buffers interfere with the Lowry method. Lowry method is compared with other methods to determine protein concentration and concludes that the advantages of the Lowry method are simplicity, sensitivity, and precision, the disadvantages are interfering substances and time compared to some of the dye-binding methods such as the Coomassie Blue methods.

Use of bicinchonic acid (BCA) was introduced as an easier way to determine protein; it uses only one reagent instead of two as in the Lowry procedure. Sensitivity is similar to the Lowry procedure, but detergents, buffer salts, and denaturing agents such as urea and guanidine hydrochloride cause less interference. However, for lipids, reducing agents, chelators such as EDTA, and acids and alkali cause interference.

The Lowry method determines both proteins, small peptides and free amino acids, while methods such as Biuret and Biorad only determine peptide chains above a certain length. However, as different amino acids and peptides give different colors in the Lowry method, the method is highly protein dependent (Table 4.1).

Method	Range (µg)
Kjeldahl	500–30,000
Biuret	1,000–10,000
Lowry	10–300
Biorad (Coomassie Brilliant Blue)	20–140
Biorad (Coomassie Brilliant Blue)—micro	1–20
Bicinchonic acid	1–50
Absorption at 280 nm	100–300

Table 3.1

Molecular weight can also be determined by electrophoresis. One of the most commonly used methods is SDS-PAGE, using gels of polyacrylamide and denaturing the samples by boiling in a solution of sodium dodecyl sulfate (SDS). SDS binds to proteins in a weight ratio of 1:1.4, which gives one SDS molecule for every two amino acids. Since SDS is charged, this results in a charged complex where the charge is proportional to the molecular

weight of the protein. Dithiothreitol (DTT) or mercaptoethanol is often added to reduce disulfide bonds. The most commonly used system is that of Laemmli. The denatured proteins are applied to the gel and an electric current is applied, causing the negatively charged proteins to migrate across the gel toward the anode. The proteins will migrate based on their size; smaller proteins will travel farther down the gel, while larger ones travel a shorter distance. By using markers of known molecular weight, a standard curve can be made in the same way as for gel chromatography and the weight of the unknown proteins determined.

3.2 Amino acids

3.2.1 Introduction

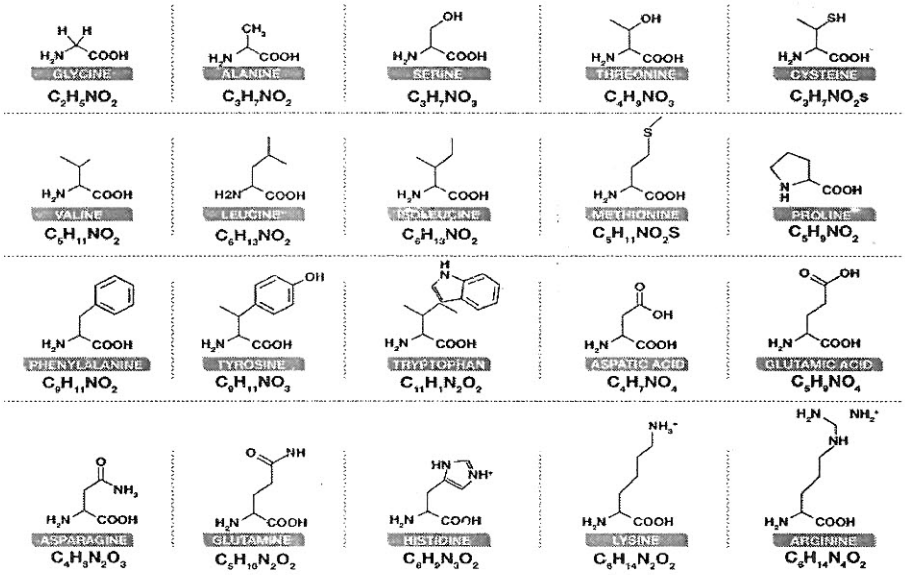

Fig. 4.1 Structure of Amino Acids

Amino acid, any of a group of organic molecules that consist of a basic amino group (—NH2), an acidic carboxyl group (—COOH), and an organic *R*group (or side chain) that is unique to each amino acid. The term *amino acid* is short for *α-amino [alpha-amino] carboxylic acid*. Each molecule contains a central carbon (C) atom, called the α-carbon, to which both an amino and a carboxyl group are attached. The remaining two bonds of the α-carbon atom are generally satisfied by a hydrogen (H) atom and the *R*group. The formula of a general amino acid is:

R – CH(NH2)COOH

3.2.2 Cation Exchange Chromatography

This methodology is based on the amino acid charge, and thus the underivatized amino acids are separated using sulfonated polystyrene beads as the stationary phase and aqueous

sodium citrate buffers as the mobile phase. The elution involves a stepwise increase in both pH and sodium or lithium ion concentration. Under these conditions, the more acidic amino acids elute first, and those with more than one primary amino group or possessing a guanidyl residue elute at the end of the chromatogram. The original method required two separate columns and needed about 4 h to achieve a complete analysis. After separation, amino acids were converted into coloured ninhydrin derivatives for spectrophotometric (colorimetric) detection.

The classical procedure has been improved with a new polystyrene matrix that offers better resolution power due to smaller particle size, speed, pellicular packaging, and better detection systems. The latest generation of amino acid analyzers also use o-phthaldialdehyde (OPA), fluorescamine, or 4-fluoro-7-nitrobenzo-2,1,3-oxadiazole postcolumn derivatization to obtain highly fluorescent derivatives with enhanced sensitivity.

3.2.3 Reversed-Phase High-Performance Liquid Chromatography

RP-HPLC has been widely used, because it requires only standard equipment that can be shared by different types of analysis. This fact and the proliferation of precolumn derivatizing agents have stimulated the development of RP-HPLC methods to analyze amino acids in all kind of matrices (food, plants, biological fluids, and tissues). Precolumn amino acid derivatization may be necessary to confer hydrophobicity to the amino acid molecule, making it adequate for partition based on chromatography, but, also, the formed molecule improves sensitivity and selectivity at the detection by allowing the spectroscopic (UV or fluorescent) detection of aminoacids. The resulting system is simpler and cheaper compared with the combination of cation-exchange plus postcolumn derivatization and permits choosing among a great number of possible methodologies, with which many of them have been marketed.

4 Fatty acids and Cholesterol

4.1 Fatty acids

4.1.1 Introduction

Fatty acid is a carboxylic acid often with a long, unbranched aliphatic chain, which is either saturated or unsaturated. Carboxylic acids as short as butyric acid (four carbon atoms) are considered to be fatty acids, whereas fatty acids derived from natural fats and oils may be assumed to have at least eight carbon atoms, e.g., caprylic acid (octanoic acid). Fatty acids are aliphatic monocarboxylic acids derived from or contained in an esterified form in an animal or vegetable fat, oil, or wax. Natural fatty acids commonly have a chain of 4–28 carbons (usually unbranched and even numbered), which may be saturated or unsaturated. By extension, the term is sometimes used to embrace all acyclic aliphatic carboxylic acids.

4.1.2 n-3 and n-6 Fatty Acids

The last two decades have seen an exponential increase of interest in the health effects of n-3 fatty acids from fish oils. Several reviews are available on their physiological roles and functions, on their tissue distribution in humans, on their structural and functional roles in cellular membranes, on their role in gestation and parturition, inflammation, immune response and autoimmunity, cortical and retinal developments, cardiovascular disease, cancer, and cellular life span.

Clinical and epidemiological studies indicated that the consumption of fish and fish oils renewed interest in investigating the lipid content and the fatty acid composition of fish and seafood products around the world. Studies were especially concentrated on the content and the availability of long-chain polyunsaturated fatty acids (PUFAs) that are typical of the aquatic habitats. Examples of PUFA include eicosapentanoic acid (EPA), and docosahexaenoic acid (DHA). The fatty acid composition of fish, shellfish, seafood products, and encapsulated fish oils products have been extensively treated and discussed.

Fish oils and marine lipids are considered to be the main source of n-3 PUFAs in the human diet. They are composed of complex fatty acids, typically an even-carbon chain length from C14 to C24 with 0–6 methylene interrupted double bonds. Marine lipids also contain minor amounts of less common fatty acids with nonmethylene interrupted and branched-chain fatty acids.

4.1.3 Analysis of fatty acids

Usually, the analysis of fatty acids in a fish tissue involves mainly three steps: lipid extraction, preparation of fatty acid derivatives, and gas chromatographic (GC) analysis. For decades, GC has been the most applied method for fatty acids analysis. The success of GC with flame ionization detector (FID) for the analysis of fatty acids is based on the ability of this technique o separate dozens of fatty acids depending on the type and the length of the column, and on the economical accessibility of the GC instrumentation that is actually present in all analytical laboratories. The advent of wall-coated open tubular (WCOT) capillary column, available in a wide range of different stationary phases, has led to an excellent resolution capability of this technique. Specific separation problems of fatty acids connected to specific applications could be solved by the alternative methodologies of sample preparation.

4.2 Cholesterol

Cholesterol is a structure containing 27 carbons, commonly found as the component in cell membrane. Biologically it is an important precursor for bile acid, provitamin D3 and several steroidal hormones. Accurate determination of cholesterol is important due to its close correlation to the occurrence of coronary heart disease. Earlier methods of cholesterol analysis rely heavily on spectroscopic and gravimetric procedures. the unsaponifiable lipid components of lipid are analysed for Cholesterol

Cholesterol reacts with ferric chloride in the presence of sulphuric acid to form a bright red coloured compound, the intensity of which is measured at 560 nm Standard calibration is prepared using pure cholesterol.

5. Vitamins

5.1. Analyses of Vitamins

Vitamins are classified according to their solubility in fat organic solvents (fat-soluble vitamins) or water (water-soluble vitamins). The solubility properties are related to the distribution of vitamins in foods as well as the analytical methods employed. Fat-soluble vitamins A, D, and E are contained in fish and seafood in varying amounts. Fatty species of fish have high concentrations of fat-soluble vitamins. Fish and seafood are good to excellent dietary sources of most of the B vitamins. Vitamins are generally susceptible to oxidation, heat, pH, moisture, light, degradative enzymes, and metal trace elements. Thus, processing, storage, preparation, and cooking methods can affect the concentrations of vitamins in seafood.

To liberate vitamins bound in lipid or protein fractions, food samples may need to be hydrolyzed using acids, alkalines, and/or enzymes or extracted directly with solvents without hydrolysis. The extract solutions may require some forms of cleanup before the vitamins are measured to remove interfering substances and to improve the sensitivity and selectivity of the analytical methods. Antioxidants such as BHT, pyrogallol, or ascorbic acid are frequently added in extraction solvents to prevent oxidation and conversion of vitamins. Most of the vitamins are liable to light, and, therefore, food samples must be protected from light during the entire analysis.

Various methodologies, including colorimetric, fluorometric, titrimetric, and spectrophotometric methods, have been developed and used for determining the vitamins in foods. However, microbiological and HPLC methods are most frequently used in estimating the vitamins in foods, because these methods have sufficient sensitivity and selectivity to quantitate low concentrations of naturally occurring vitamins. Microbiological assay can be applied to all the B vitamins. Lactic acid bacteria are suitable for determining the B-vitamins turbidimetrically, except for vitamin B6, which may be determined using yeasts. Fat-soluble vitamins and vitamin C are most commonly determined by HPLC. HPLC methods can also be used for estimating the B vitamins. HPLC distinguishes between naturally occurring and added (fortified or enriched) vitamins and also separates the individual forms of vitamins. Some vitamins, with low UV absorbance or fluorescence responses, can be determined after conversion to fluorescent derivatives using pre- or postcolumn derivatization. Bio-specific methods for determining some of the water-soluble vitamins include immunoassays and protein-binding assays. Newer techniques continue to be developed for quantitating the concentrations of the various vitamins in all types of foods, including fish and seafood.

6. Minerals and Trace Elements

6.1 Minerals

Minerals are inorganic elements necessary in the diet for normal body functions. They can be divided into two groups (macro-minerals and micro-minerals) based on the quantity required in the diet and the amount present in fish. Common macro-minerals are sodium, chloride, potassium and phosphorous. These minerals regulate osmotic balance and aid in bone formation and integrity. Micro-minerals (trace minerals) are required in small amounts as components in enzyme and hormone systems. Common trace minerals are copper, chromium, iodine, zinc and selenium. Fish can absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet.

Fish contains most of the 90 naturally occurring elements. The average ash content in the edible part of the fish may range from 0.5-1.8% and it is an indication of total minerals. Under mineral profiling the determination of alkali metals, *viz*; Na, K, and Ca are presented here and are normally determined by flame photometer method

6.2 Trace elements

Among the various pollutants, trace metals and metalloids, if occurring in higher concentrations, can become severe poisons for all living organisms due to their high persistence, toxicity and tendency to accumulate in water and especially in sediment. Since heavy metals are non-biodegradable, they can be accumulated by various aquatic organisms including fish. There are two main routes of trace elements uptake for fish: aqueous uptake by the gills (and to a lesser extent by skin) and dietary uptake by ingestion of contaminated food. Being the top predators in aquatic food chains, fish have been widely used as bioindicators of metal pollution. Although trace elements accumulate in different fish organs (liver, kidney, muscle, gills), fish muscle is the most frequently used tissue for the analysis because it is the main edible part of the fish.

The presence of trace element from anthropogenic origin in marine ecosystems has been a serious problem for the environment and human health. Their intake can lead to adverse health effects like renal dysfunction, lung disease, liver failure, dysfunctions in the kidneys and chronic damage to the central and peripheral nervous system

The world consumption of fish has increased simultaneously with the growing concern of their nutritional and therapeutic benefits. The content of toxic Trace elements in fish can counteract their beneficial effects. Atomic absorption spectrophotometry is normally used for the analysis of Trace metals.

In Flame atomic absorption spectrophotometry, a sample is aspirated in to a flame and atomized. A light beam is directed through the flame, into a monochromator, and on to a detector that measures the amount of light absorbed by the atomised element in the flame. As each metal have its own characteristic absorption wavelength, a source lamp composed of that element is used, which makes the method relatively free from spectral and radiation interferences. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of the element in the sample over a limited concentration range (linear range).

7. Environmental Contaminants: Persistent Organic Pollutants

7.1 Analyses of Persistent Organic Pollutants

Persistent organic pollutants (POPs) are organic compounds of natural or anthropogenic origin characterized by low water and high lipid solubility, resulting in bioaccumulation in fatty tissues of living organisms. They are widespread contaminants of the marine ecosystem mainly because of their depositions from the atmosphere, able to be transported for long distances from their point of origin, or as result of river wastewater transport, surface runoff, industrial development, or agricultural activities. It is important to highlight that all pollutants, whether in air or on land tend to end up in the ocean; furthermore, closed or semi enclosed seas are particularly exposed to the pollution risk. In recent years there has been a growing interest in these pollutants, in particular for their impact on human health. The risks posed by POPs for human health have become of increasing concern and are actually object of a worldwide agreement among several governments, including measures to reduce or eliminate their release in the environment. Their environmental presence is of particular gravity because of their toxicity, bioavailability, and persistence. The seas and coastal areas are, generally, final recipients for terrestrial wastewaters containing both anthropological and natural origin pollutants; furthermore, at the same time they are very important economic and aquatic resources. POPs' presence poses serious adverse effects on the marine ecosystem because they affect all organisms from primary to secondary producer levels up until the top levels of the seafood chain. Pesticides, Polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) have been listed as priority pollutants by the United Nations Environment Programme (UNEP) because of their potential carcinogenicity, mutagenicity, and toxicity to aquatic organisms and humans.

Pesticides include both organochlorine and organophosphorous compounds, their detection at ppb levels require mass spectrometric methods. Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds consisting of at least two or more fused aromatic rings of carbon and hydrogen atoms. The chemical properties of PAHs depend on their number of rings and molecular mass. In general they are solids having high melting (60°C–450°C) and boiling (200°C–600°C) points, showing low degrees of volatility, and are rather inert lipophilic

compounds, which easily dissolve in organic solvents. Chromatographic techniques, such as high performance liquid chromatography (HPLC) and gas chromatography (GC) are common methods of PAHs detection.

PCBs constitute a family of environmental persistent pollutants of synthetic organic compounds that have mainly been used in electrical equipment as dielectric insulating media. The use of these compounds is now restricted, but because of their wide usage in the past and of their high stability in the environment they are so widely distributed that detectable levels can be found in marine organisms, from mollusks to fish. Determination of PCBs in marine organisms generally consists of three steps: sample extraction, purification, and chromatographic separation, identification, and quantification. The extraction methods are generally set up to maximize the extraction of all analytes and the clean-up is performed to improve the selectivity of the extraction removing lipids and interfering compounds. Presently the most widely practiced technique for the PCB determination is capillary GC–ECD. The use of mass spectrometric detectors will enhance selectivity.

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