20 Fatty Acids

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20.1 INTRODUCTION

Lipids are ubiquitous components of all living organisms in both terrestrial and aquatic environment. Fatty acids (FAs) are the chief constituents of lipid molecules, exploiting essential functions for the formation of cells and tissues as structural components of membranes, metabolic energy sources, and chemical messengers [1].

Seafood and aquaculture products, including fish, molluscs, and crustaceans, are on the focus of the scientific community because of their valuable nutritional composition, considered as a balanced source of several nutrients difficult to attain in any other food of animal origin [2]. Particularly, fish lipids are considered the richest natural source of n-3 long-chain polyunsaturated fatty acids EPA and DHA [3], known to own potential beneficial effects on consumers health, above all carrying cardiovascular benefits, defence from incident Alzheimer's disease and fundamental activities during growth and development of individuals [4].

Scientists estimate that the beneficial effects associated with fish and other seafood consumption, particularly when consuming a variety of them, outweigh the potential risks related to the ingestion associated with the presence of toxins, allergens, chemical pollutants, and so on [4, 5, 6]. It has to be considered that the amount of nutrients encompassed in seafood (including FAs), as well as contaminants, can vary significantly in relation to both inner factors (fish species, age, physiological stage, fat content, etc.) and external factors (environmental conditions, season, trophic level, food availability, processing treatments, etc.) [6]. Thus, since a variety of fish species are consumed across Europe, the European Food Safety Authority (EFSA) did not provide general recommendations on fish consumption based on a risk-benefits approach, suggesting that each country should make such evaluations considering its own pattern of fish consumption [7]. Overall, EFSA stated that dietary recommendations for EPA and DHA based on cardiovascular risk considerations for European adults are between 250 and 500 mg/day [8]. Due to the high EPA and DHA content, even small increases in fish consumption are considered to have great potential impact on public health in terms of cardiovascular diseases reduction, with one to two fish serving per week (one of which to be oily) suggested to be optimal in a healthy diet [9].

20.2 LIPIDS AND FATTY ACIDS

20.2.1 Structure, Classification, and Nomenclature of Fatty Acids

Fatty acids (FAs) are aliphatic monocarboxylic acids naturally characterised by carbon chain from 4 to 28 carbon atoms [10], which may be saturated or unsaturated, straight, or branched. Conventionally, FAs can be classified based on the following:

Carbon chain length: FAs can be divided into short-chain fatty acids (SC-FAs) when the carbon chain is <C6; medium-chain fatty acids (MC-FAs) when the carbon chain is C6–C12; long-chain fatty acids

(LC-FAs) when the carbon chain is C13–C21; very long-chain fatty acids (VLC-FAs) when the carbon chain is >C22 [11].

Carbon chain structure: FAs can be divided into even- or odd-chained and straight- or branched-chained. Most of naturally occurring fatty acids are even and straight-chained. However, many dairy products and fish oils are also natural sources of branched chain fatty acids (BCFAs), being among the dominating fatty acids in the lipid bilayer of several bacteria, even if their concentration in foodstuffs is generally low (1%–3% of total lipid content) [12]. Branched-chain fatty acids (BCFAs) can be further divided into their iso and anteiso forms, when the ramification is placed on the second-to-last and third-to-last carbon counting from the terminal methyl carbon, respectively. Commonly, BCFAs are named indicating the total number of carbon atoms in the chain and the position of the branch. For example, iso16 (IUPAC name: 14-methylpentadecanoic acid) indicates a 16-carbon atoms carboxylic acid with a methyl ramification of the chain placed on the second-to-last carbon atom counting from the terminal methyl group (thus, the 14th carbon atom counting from the carboxylic group).

Unsaturation degree: FAs can be divided into saturated fatty acids (SFAs) when the carbon chain does not contain any double bond between carbon atoms; monounsaturated fatty acids (MUFAs) when the carbon chain contains one double bond between carbon atoms; polyunsaturated fatty acids (PUFAs) when the carbon chain contains ≥2 double bonds between carbon atoms. The double bonds in PUFAs are generally methylene-interrupted (-CH2-), with the exception of conjugated fatty acids. Common nomenclature used for PUFAs is given indicating the length of the carbon chain, the number, and the position of double bonds. For this purpose, the “n” (or “ω”) notation is commonly used when all the double bonds are in cis configuration; in this case, the position of the first double bond counting from the methyl terminal carbon is indicated (i.e. linoleic acid is 18:2n-6 or 18:2ω-6). At the same time, the “D” notation can be used, which indicates the position of all double bonds, but counting from the carboxylic group (i.e. linoleic acid is: D9,12–18:2). Additionally, in this notation, also the stereo-isomeric conformation of double bonds can be indicated (i.e. linoleic acid is cis-9,cis-12, 18:2).

Trivial or common names are usually used by scientists, adding the suffix “-ic” to a root indicative of the natural source or some property of the fatty acid [13]. An example including the nomenclature of polyunsaturated fatty acids commonly found in seafood and aquaculture products is reported in Table 20.1.

TABLE 20.1
Nomenclature of Polyunsaturated Fatty Acids Commonly Found in Seafood and Aquaculture Products

Fatty Acid	Systematic Name (Δ-Notation)	Trivial Name
C18:2n-6	c9,c12-Octadecadienoic acid	Linoleic acid
C18:3n-6	c6,c9,c12-Octadecatrienoic acid	g-Linoleic acid
C18:3n-3	c9,c12,c15-Octadecatrienoic acid	α-Linoleic acid
C18:4n-3	c6,c9,c12,c15-Octadecatetraenoic acid	Stearidonic acid
C20:2n-6	c11,c14-Eicosadienoic acid	Eicosadienoic acid
C20:3n-6	c8,c11,c14-Eicosatrienoic acid	Dihomo-g-linolenic acid
C20:3n-3	c11,c14,c17-Eicosatrienoic acid	Eicosatrienoic acid
C20:4n-3	c8,c11,c14,c17-Eicosatetraenoic acid	Eicosatetraenoic acid
C20:4n-6	c5,c8,c11,c14-Eicosatetraenoic acid	Arachidonic acid
C20:5n-3	c5,c8,c11,c14,c17-Eicosapentaenoic acid	EPA
C22:2n-6	c13,c16-Docosadienoic acid	Docosadienoic acid
C22:3n-3	c13,c16,c19-Docosatrienoic acid	Docosatrienoic acid
C22:4n-6	c7,c10,c13,c16-Docosatetraenoic acid	Adrenic acid
C22:5n-3	c7,c10,c13,c16,c19-Docosapentaenoic acid	DPA
C22:6n-3	c4,c7,c10,c13,c16,c19-Docosahexaenoic acid	DHA

20.2.2 Lipid Fractions

In biological systems, FAs can be found in either free or bonded form. In the latter case, they are esterified to other molecules, mostly glycerol, as in the case of phospholipids and glycerolipids [11,14]. In aquatic organisms, the most of lipids are acylglycerols, in which FA are esterified to a glycerol backbone: triacylglycerols (TAGs), phospholipids (PLs), and glycolipids (GLs). Free fatty acids (FFAs) are present at very low concentrations in living animals [1], but they can be released by enzymatic action after fish death through lipolysis of TAGs and PLs, a process that can persist even during frozen storage of seafood and aquaculture products [15]. The accumulation of FFAs during processing and storage can affect negatively the quality and the shelf-life of the final product, causing textural alteration due to the interaction of FFAs with proteins, decreasing their solubility, and leading to organoleptic deterioration [15,16].

20.2.2.1 Neutral Lipids

Neutral lipids mainly consist of storage lipids such as TAGs and wax esters (WE); minor compounds belong to the group of neutral lipids, such as mono- (MAGs) and di- (DAGs) acylglycerols, FFAs, sterols (free and esterified), and fatty alcohols (ALCs).

The most (~99%) of food lipids are represented by TAG as storage lipids. They consist of a glycerol backbone in which the hydroxyl groups are ester-linked to different fatty acids; the properties (physical, texture, rheological) of TAGs depend on their FAs composition and distribution among the position in the glycerol molecule (sn-1, sn-2, sn-3) [17]. DAGs and MAGs contain 2 mol and 1 mol of fatty acid per mole of glycerol, respectively, and are present in lower concentrations in animal tissues, even if they can be formed as intermediates during the biosynthesis or the hydrolysis of TAGs [18]. Storage fat (mainly, TAGs) in animals contain relatively high amounts of SFAs (mainly, 16:0) and MUFAs (mainly, 18:1), even if significant differences are detectable between different species, with fish containing high amounts of C20 and C22 FAs in the adipose tissue [18]. Storage lipids in non-ruminants mainly reflect the FAs profile of the consumed food being incorporated in this fraction almost unaltered [19].

20.2.2.2 Polar Lipids

Polar lipids mainly represent structural lipids contained in cellular membranes, such as glycerophospholipids (PLs), glycolipids, and sphingolipids. PLs are have a glycerol backbone with two fatty acids esterified at the sn-1 and sn-2 positions and a phosphate ester at the sn-3 position in a bond to organic bases or other moieties [20]. Based on the nature of such moiety (choline, ethanolamine, serine, inositol, glycerol), we can differ between phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phisphatidylglycerol (PG), and phosphatidylinositol (PI) [21]. The most abundant lipids in the cellular membranes of animal tissues are PCs and sphingomyelin (SM), followed by minor components, such as lysophosphatidylcholine (LPCs) and phosphatidylethanolamines (LPEs), which contains only one fatty acid, PSs and PIs [18]. In animals, the sn-1 position of PCs is largely esterified by saturated or monounsaturated fatty acids, while the sn-2 position contains polyunsaturated fatty acids [17,22].

In polar lipids, polarity is conveyed by the presence of a hydrophilic head group, which allows for the formation of lipid bilayers in membranes [1]. The nature of the polar head group and the linkage with the aliphatic chain have an influence on the properties of the lipid molecule, affecting both the stability and fluidity of membranes and the ability of the lipid molecule itself to work as membrane receptor [23]. Due to the necessity to accomplish this set of biological functions, polar lipids generally contain substantial proportions of the longer-chain PUFAs [18]. Not only the nature but also the positional distribution of different FAs in lipid molecules varies between tissues and has some metabolic importance [18]. Actually, the phospholipid form can augment the stability, the bioavailability, and therapeutic effectiveness of the bioactive n-3 VLC-PUFAs, EPA, and DHA, compared with TAG; mostly, only when linked in a PL molecule, these FAs can cross the blood-brain barrier [20,21,24,25].

20.3 FATTY ACIDS AS BIOACTIVE COMPONENTS

The first role of FAs in eukaryote organisms is structural. They represent the “building blocks” of cell membranes, regulating fluidity, the response to temperature stress and optimising position, amount and function of membrane proteins [3,14]. The functional properties of FAs in cell membranes, as constituent parts of membrane lipids, are strictly dependent by their structure: chain length, degree of unsaturation, double-bond position, and hydroxylation. More in details, the longest and the more unsaturated the aliphatic chain of FAs, the more fluid the membranes, with PUFAs associated with the highest membrane fluidity [3,26].

A further role of FAs in eukaryotes is their bioactivity. First, FAs are energy suppliers; second, they are signalling molecules and fundamental precursors of hormones and other bioactive lipid mediators [3,14,27].

SCFAs and MCFAs represent key metabolites in energy metabolism and anabolic processes in mammals. They show regulatory and signalling functions in cell metabolism and have been indicated to play fundamental roles in the pathogenesis of several diseases [28].

At the same time, unsaturated fatty acids (UFAs) own several beneficial effects on consumers' health. For example, oleic acid (OA, 18:1n-9) has positive effects towards the digestive and cardiovascular systems and the inflammatory response, and plays a pivotal role in the management of the oxidative stress and in the development of the nervous system [29]. Long-chain PUFAs (LC-PUFAs) modulate intracellular signalling and regulate functions within immune cells (neutrophils, monocytes, macrophages, dendritic cells, T cells, B cells) [30]. Moreover, the VLC-PUFAs, arachidonic acid (ARA, 20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), serve as precursors for the generation of bioactive lipid mediators (eicosanoids, docosanoids, prostaglandin, thromboxanes, leukotrienes) related to different roles during inflammatory processes and cardiovascular health [3,31,32]. In details, high contents of ARA in membrane phospholipids are associated with excess pro-inflammatory cytokine production, while high contents of EPA are associated with an inhibition of the pro-inflammatory cytokines and to the generation mediators that improve vasodilation and decrease inflammation and aggregation [33,34].

Generally, n-6 and n-3 PUFAs show opposite physiological functions on cardiovascular diseases, cancer, autoimmune diseases, and neural development, with n-3 FAs (mostly, EPA and DHA), representing key bioactive components associated with positive effects on health status [35]. A correct balance between FAs of the n-6 and n-3 series in human diets, represented by an n-6/n-3 ratio as lower as possible (with the optimal ratio ranging from 1/1 to 4/1), is considered fundamental to assure their biochemical efficiency, while typical modern Western diets reach n-6/n-3 values around 15–20/1 [36]. Lipids can be classified in polar and non-polar (or neutral). Fatty acids are distributed among both the lipid classes, representing an essential and integral part of the whole lipid complexes [37].

20.4 LIPIDS AS MARKERS OF ORIGIN

Lipid composition of seafood and aquaculture products is a topic of extreme interest in the scientific community due to the known nutritional properties of specific fatty acids characteristic of fish oils, related to positive biological activity on consumers health. The consumption of fish has been associated with a reduction in risk of developing many inflammatory and/or chronic diseases (such as high blood pressure, strokes, rheumatoid arthritis, etc.) [38].

Among the whole complex of macro- and micro-nutrients, many FAs, such as the n-3 VLC-PUFAs, play a pivotal role in conveying positive functional properties to seafood products. Many aquatic organisms, such as algae, protists, and bacteria, are the main source of natural n-3 VLC-PUFAs, being capable to synthetise them through specific enzymes. Differently, fish (as vertebrates) are only able to desaturate and elongate their precursor, alpha-linolenic acid (ALA, 18:3n-3), into the longer n-3 FAs at low rates (8% for EPA and 1% for DHA) [14,21,39]. Furthermore, some marine fish are even unable to elongate and desaturate ALA into EPA and DHA, thus these species utterly need to introduce the n-3 VLC-PUFAs by their diet [40].

FAs synthetised by primary producers in the aquatic environment enter into the aquatic food web and are transferred to upper trophic levels, up to both herbivorous and predatory fish [19,21,41, 42, 43, 44]. Therefore, lipids derived from fish and other seafood products are characterised by high amounts of n-3 VLC-PUFAs and, consequently, very low n-6/n-3 ratios, not exceeding 1/1 for most wild species [21]. Entering in the human food chain, they play a fundamental role in regulating the FAs balance in diets. Furthermore, marine organisms at the lower levels of the aquatic food web (such as krill feeding on marine phytoplankton) and many aquatic products (such as fish roes) contain considerable amounts of PLs [25,45], and as seen

before, PLs are fundamental sources of highly bioavailable n-3 VLC-PUFAs with recognised beneficial effects on consumers' health [21,24].

In both freshwater, estuarine, coastal, and deep-sea environments in all latitudes, other than being influenced by fish diet, FAs composition of aquatic organisms varies according to inner (phylogeny, developmental/reproductive stages, age and size of fish, etc.) and external (water temperature, salinity, hydrostatic pressure, etc.) factors [1,46,47]. In this context, many FAs can be considered important biomarkers, whose presence is linked to variations of both physiological and environmental conditions. FAs signature of tissues of aquatic organisms can be a potential tool to provide information about substantial ontogenetic changes in different species, due to both dietary changes and internal regulatory processes (related to changes in energy requirements and FAs bioconversion ability) [48, 49, 50]. FAs profiling in seafood and aquaculture products has been employed as a potential tool to provide information about the origin of the product in terms of animal species, production system, and, in case of aquaculture products, even feeding strategy [51, 52, 53, 54, 55, 56]. Actually, FAs distinctive of vegetal raw materials commonly included in aquafeed formulations (seeds, cereals, vegetable oils, etc.) are considered good indicators for the discrimination between wild and farmed specimens, with linoleic acid (LA, 18:2n-6) as the most characteristic [57,58]. When vegetable oils are included in farmed fish diets, fish fillets show an increase in the content of C18 PUFAs, mainly LA, a decrease in the n-3 series VL-PUFAs and changes in the composition of MUFAs [57].

Finally, different FAs can be selectively detected in different lipid fractions. It is known that since the first developing stages of fish (embryos and larvae), SFAs and MUFAs are mainly catabolised to provide energy, while PUFAs tend to accumulate in vitellogenin because of their fundamental biological functionality in the development of the new individual [59, 60, 61]. Among MUFAs, the mostly represented in fish, oleic acid (OA, 18:1n-9) is indicated as the most abundant regardless of the origin and the species, representing the main source of energy for aquatic animals since the embryonic stages [62]. Because of that, OA mainly accumulates in storage lipid tissues, such as the yolk oil droplets in eggs [62,63] and the adipose tissues in adult animals [37]. Otherwise, the n-3 series VLC-PUFAs, EPA and DHA, represent essential FAs for larvae development, as structural component of cell membranes and precursors of prostaglandins and eicosanoids in fish eggs [62]. Consequently, they are mostly detected in the phospholipid fraction of eggs, independently by the species [63, 64, 65, 66, 67, 68].

20.5 ANALYTICAL DETERMINATION OF FATTY ACIDS

The analytical technique most widely employed for fatty acids profiling in seafood, aquaculture products, and fish oils is represented by gas chromatography (GC). Prior to GC, several steps are performed in order to extract lipids from samples and make FAs suitable for the analysis.

Since lipids are highly susceptible to hydrolysis and oxidation induced by light, high temperatures, oxygen, and enzymatic processes, particular care should be kept during the entire process of analysis, in order to avoid lipid degradation. Therefore, appropriate handling conditions, including adequate and rapid storage at freezing temperatures (with possible addition of antioxidants and/or storage under a nitrogen atmosphere or vacuum), should be observed in order to control sample degradation and consequent analytical biases [1,69,70].

20.5.1 Lipid Extraction

The first step for FAs analysis by GC is the quantitative and exhaustive extraction of total lipids from the sample and the removal of any non-lipid molecules, such as amino acids, entire proteins, and saccharides. Lipids are linked to other molecules by weak hydrophobic forces, by hydrogen bonds and by ionic bonds [70].

In order to break these bonds and to extract total lipids from a food matrix, lipid molecules must be dissolved in appropriate solvents that can penetrate the sample particles, whose size must be as small as possible [1]. In order to achieve this goal, samples usually are blended and/or grinded before the extraction.

Lipids from seafood and aquaculture food comprise a wide range of molecules characterised by different polarity. Non-polar lipids are very soluble in hydrocarbon solvents, such as hexane, cyclohexane, or toluene, and in low-polarity solvents, such as chloroform, or ethers. On the contrary, polar lipids are highly soluble in more polar solvents, such as methanol and ethanol [71]. For this reason, the most of the methods developed and employed up to date for total lipid extraction are based on the use of mixtures of organic solvents characterised by different polarity. The gold standard for the extraction of lipids in aquatic products is still represented by the employment of a mixture of chloroform/methanol (2/1), as introduced in the late 1950s by Folch et al. [72] and Bligh and Dyer [73]. Both the methods are based on the establishment of a tertiary system formed by chloroform, methanol, and water in certain proportion, in order to achieve a high recovery of all lipid species present in fish tissues. The Bligh and Dyer method, that is only applicable when the sample matrix contains almost 80% endogenous water, has the advantage to allow the employment of lower solvent amounts and, consequently, to be more sustainable by an environmental and a safety point of view. Nowadays, the academic community is making efforts in order to reduce the health and environmental impact of analytical practices, encouraging the exploitation of alternative organic solvents and novel eco-friendly methodologies for lipid extraction [1]. In this context, since chloroform is characterised by high toxicity, several methods have been developed with the aim to substitute this solvent with others represented by the same extraction efficiency. Such an example, dichloromethane was demonstrated to be suitable for use in substitution of chloroform for lipid extraction and fatty acid assessment, thus avoiding the major health and safety issues associated with the use of chloroform [74]. Other solvents, such as methyl-tert-butyl ether [75, 76, 77, 78] or butanol/methanol mixtures [79], have been evaluated as extraction solvents, allowing to achieve high extraction recoveries and representing more environment-friendly and less toxic alternatives to the traditional techniques. However, the Folch and Bligh and Dyer protocols are still considered as the first choice for lipid extraction in fish and seafood matrices [11].

Novel, fast and sustainable methods for lipid extraction could be discovered and developed thanks to the employment of recent and advanced technologies. Such an example, a microwave-assisted extraction (MAE) technique was developed and validated on fish matrices with satisfactory results [80,81], reaching total lipid content and individual FAs amounts comparable to those obtained by means of the Folch and the Bligh and Dyer extraction methods. Even if some concerns exists about the stability of the lipids during microwave treatment, many authors reported satisfactory results, with MAE requiring minimum sample handling and providing fast and reliable extraction minimising FAs peroxidation [82].

Similarly, Dodds et al. [83] developed a micro-scale lipid extraction of fish tissues (100 mg) using accelerated solvent extraction (ASE), reaching quantitative and qualitative efficiency as conventionalliquid–liquid methods. The ASE technique is based on the controlled increase in solvent temperature and pressure during extraction, maintaining solvents close to their supercritical region where they have high extraction properties, thus allowing the employment of low solvent volumes and short extraction times [1].

20.5.2 Lipid Fractions Separation

By comparing the relative FAs proportion in different lipid fractions may help the identification of the dietary FAs selectively retained or eliminated for physiological requirement and in response to stress stimuli or dietary changes [61]. For all these reasons, the separation of individual lipid classes prior to their analysis can be fundamental to address trophic ecology, nutrition, and comparative physiology issues.

Since the 1980s, lipid chemists put an effort in the development and the optimisation of appropriate techniques to correctly separate the classes included in a whole lipid extract. Nowadays, the most investigated and employed technique for lipid classes separation is the solid phase extraction (SPE). A SPE method for lipid classes separation was first reported by Kaluzny et al. [84], then it was modified and applied on several food matrices, including seafood and seafood products [12,85], allowing the fractionation of a number up to eight lipid classes (mono-, di-, and tri- acylglycerol, cholesterol, steryl esters, free fatty acids, phosphatidylcholine, and phosphatidylethanolamine).

The SPE process simply implies a physical extraction process involving a liquid and a solid phase, the latter represented by commercial pre-packed columns containing stationary phases that can be related to those used widely in liquid chromatography. The packing material is held in a place within a plastic column, connected to a vacuum manifold. The principle of the separation depends on the nature of the stationary phase, involving the mechanisms of adsorption, partition, or ion-exchange chromatography. The isolation of individual simple lipid classes is generally performed by stepwise elution from columns using different organic solvents, depending on the degree of polarity of each lipid class [86]. According to the original method of Kaluzny et al. [84], the lipid fractions were recovered using the solvent combination described in Table 20.2. The original method was often modified and optimised for individual applications, as in the case of Pinkart et al. [87], reported in Table 20.2. A better separation into major lipid classes and a more accurate FAs profile determination in lipid fractions can be obtained when optimising the standard method for the matrix under analysis [88].

TABLE 20.2
Lipid Fractionation System by 100 mg Aminopropyl-SPE Cartridges as Optimised in Muscle Foods [88]

Lipid Fraction	Solvent System	
	Kaluzny et al. [84]	Pinkart et al. [87]
Neutral lipids	Chloroform/2-propanol (2/1) 3 mL	Chloroform 5 mL
Free fatty acids	Acetic acid/diethyl ether (2/98) 3 mL	Acetic acid/diethyl ether (2/98) 5 mL
Phospholipids	Methanol 3 mL	Methanol/chloroform (6/1) 2.5 mL +0.05 M methanol/chloroform (6/1) 2.5 mL

The solvent amounts reported in the table were applied in 100 mg of intra-muscular fat.

Besides, both the methods provided a further separation of neutral lipids in subclasses (cholesteryl esters, triglycerides, cholesterol, diglycerides, and monoglycerides) as reported in Table 20.3.

TABLE 20.3
Solvent System Employed for the Separation of Neutral Lipids in Subclasses by Aminopropyl SPE Cartdriges According to the Methods Developed by Kaluzny et al. [84] and Pinkart et al. [87]

Neutral Lipids Class	Solvent System	
	Kaluzny et al. [84]	Pinkart et al. [87]
Cholesteryl esters	Hexane	
Triglycerides	Diethyl ether/methylene chloride/hexane (1/10/89)	Chloroform/methylene chloride/hexane (2/10/88)
Cholesterol	Ethyl acetate/hexane (95/5)	
Diglycerides	Hexane/ethyl acetate (15/85)	
Monoglycerides	Chloroform/methanol (2/1)	

Commercial SPE columns are available with a wide range of chemically bonded stationary phases, each of them associated with great potential for the isolation of specific lipid classes [1]. For example, the Kaluzny method was built using aminopropyl-bonded silica gel column [84].

During last years, the SPE method increasingly replaced the oldest thin-layer chromatography (TLC) methods, where the separation of individual complex lipid classes occurred based on the different migration characteristic in a single dimension [89]. However, among the analytical techniques suitable for lipid classes separation to date, high-performance liquid chromatography methods combined with high-throughput detection systems are the favoured ones, as widely illustrated in the Section 20.6 of this chapter.

20.5.3 Analysis of Fatty Acids Derivatives by Gas Chromatography

Fatty acids included in the total lipids or individual lipid fractions of food matrices are mostly analysed by gas chromatography (GC). GC is a separation technique based on the partition processes of the analytes present in a complex mixture between the particles of a chemical-bonded support, the stationary phase, and a flowing solvent, the mobile phase. In GC, the mobile phase is represented by an inert carrier gas and the stationary phase consist in a capillary column composed by a thin layer of liquid covering a solid support (commonly made of synthetic fused silica) [90]. Nowadays, commonly employed columns are typically characterised by lengths between 30 and 100 m, a film thickness variable between 0.1 and 5 µm, and inner diameters (ID) of 0.10–0.53 mm [91].

Since the mobile phase is in the gaseous phase, FAs must be chemically modified (derivatised) with the aim to make them volatile to be analysed by GC. The derivatisation methods most known for this purpose are the hydrolysis of lipids and the preparation of esters of FAs [71]. Nowadays, the preparation of fatty acids methyl esters (FAMEs) represents the derivatisation reaction most cherished for FAs analysis by GC [1,11,92,93].

The preparation of FAMEs can be performed by means of both acid- and base-catalysed reactions. Acid-catalysed methods can be useful for the derivatisation of FAs from total lipids, allowing both the esterification and the trans-esterification of FFAs and esterified FAs, respectively [71]. The acid-catalysed derivatisation has the drawback to potentially alter the isomer distribution of the conjugated FAs [94,95]. However, fish products are not enriched in this group of FAs (such as the conjugated linoleic acid isomers), thus this derivatisation technique is widely and safely employed for lipid analysis in seafood and aquaculture products. On the other hand, basic-catalysed derivatisation methods have the potential to be fastest and to avoid the isomerization issue, but their derivatization capacity in biological matrices is limited to esterified FAs, thus exclusively allowing the trans-esterification of bonded FAs [71]. Both the acid- and base-catalysed reactions are performed in the presence of excess of methanol as methyl-group donor for methyl esters formation [96], and different catalysts can be used with each one having their own advantages and limits. For example,

among the acid catalysts we can list are hydrogen chloride, sulphuric acid, and boron trifluoride while among the base catalysts are sodium and potassium methoxide [71].

Several alternatives to the traditional extraction and derivatisation protocol have been reported during last years. For example, Kang et al. [97] developed a simplified method for the analysis of PUFAs represented by a technique that combined the extraction and the methylation into a single step. The authors demonstrated that the lipid extraction step, prior to methylation, could be omitted when the target of the analysis is long-chain (>C18) PUFAs, while it is still mandatory for the quantification of medium- and short-chain (<C16) FAs. After the derivatisation step, FAMEs are extracted in a GC-compatible solvent, and the extract is washed several times to remove any acid and glycerol residues before the injection in the chromatographic system [71].

In modern technologies, the separation of FAMEs is performed in capillary column inserted in the oven chamber of the GC equipment, characterised by physical and chemical characteristics (length and nature of the stationary phase) specifically designed based on the type of FA to be investigated. According to the basic principles of chromatography, the longer the column the higher the peak resolution power.

The GC columns most used for FAMEs separation in aquatic samples are highly polar columns, which allows for an optimal separation of FAMEs based on the carbon chain length, the number, and the location of double bonds, and especially, the configuration of the unsaturated bonds (cis and trans isomers) [1]. With polar columns, the retention of FAMEs in the stationary phase increases with their boiling point; consequently, the elution time increases with the chain length and with the unsaturation degree. For example, in Figure 20.1, the elution region of C18 FAMEs of a commercial mixture (37 Component FAME Mix by Supelco) performed by means of a TRACE™ TR-FAME GC column (120 m × 0.25 mm × 0.25 µm) by Thermo Fisher Scientific is reported.

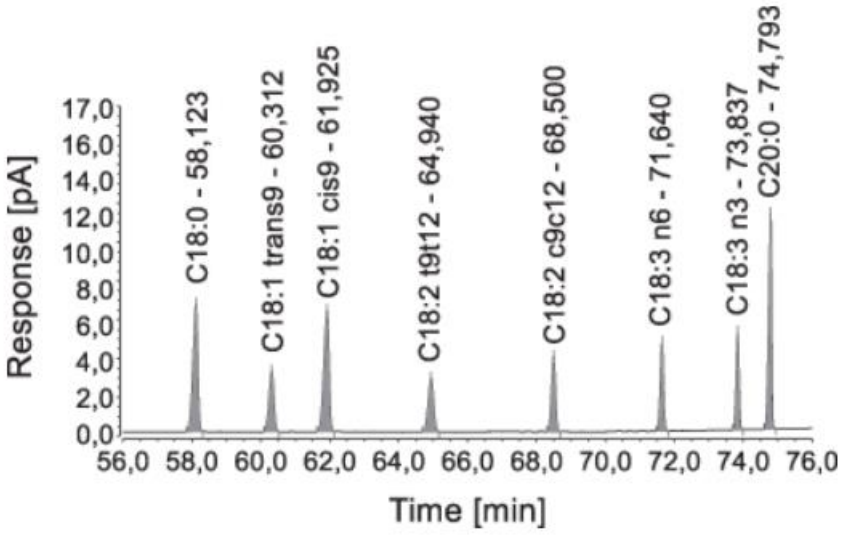

FIGURE 20.1 C18:0-C20:0 FAMEs elution region of a commercial mixture (37 Component FAME Mix by Supelco) performed by means of a TRACE™ TR-FAME GC column (120 m × 0.25 mm × 0.25 µm) by Thermo Fisher Scientific. The oven temperature was a customised temperature ramp described in Lopez et al. [98]. Image by the authors.

Different producers can provide capillary columns characterised by slightly different or completely equivalent stationary phases specifically designed for FAMEs analysis. Some examples of high-polarity columns suitable for FAMEs separation by GC are reported in Table 20.4.

TABLE 20.4

Commonly Spread Chromatographic Columns Employed in FAMEs Analysis in Food, According to the USP (US Pharmacopoeia) Classification [99]

G14, G15, G16, G20, G39, G47 (*Polyethylene Glycol, PEG*)	
AT-WAX	MEGA-WAX
BP20	MEGA-WAX 400/1000
CA-AquaWax	MEGA-WAX 20M
Carbowax 20M	Meta.WAX 400
Carbowax 400/540/1000/1500/1540/3350/4000/8000	Omegawax
CA-Wax	Optima WAX (plus)
Ciola-Wax (20M)	Permabond CW20-M (DEG)
Ciola-Wax-400/1000/3500	Quadrex 007-CW
CP-Carbowax 400	Quadrex BTR-CW
CP-Wax 52 CB	Rtx-Wax
CS-Wax	Sapiens-WAX.HT (MS)
CW-20M	SH-Rtx-Wax
DB-FATWAX UI	SH-Stabilwax
DB-HeavyWAX/DB-Wax(etr)/DB-WAX UI	SolGel-Wax
EC-WAX	Stabilwax
Elite-WAX (ETR)	Stabilwax-DB/-MS
FS CW 20 M-AM	SUPELCOWAX-10
GsBP-Carbowax 20M	TRACE TR-Wax(MS)
GsBP-InoWax(MS)	TRB-WAX
GsBP-Wax	Ulbon HR-20M
HI-WAX(MS)	VF-WAXms
HI-WAX20M	WEL-PEG20M
HI-WAXHT	WM-CarboWax 20M
HP-INNOWax	WM-INOWAX
InertCap Pure-WAX	WM-PEG20M
InertCap WAX/WAX-HT	Zebron ZB-WAX(plus)
Inno Wax	

G7 (50% 3-cyanopropyl, 50% phenylmethylsilicone) **G19** (25% phenyl, 25% cyanopropyl, 50% methylsilicone)	
BP225	OV-225
AT-225	Quadrex 007-225
CA-225	Rt-2300
Ciola-11/2225	Rtx-225
CP-Sil 43 CB	SH-Rtx-225
DB-225(ms)	Silar-5CP
Elite-225	Silicone OV-225
GsBP-225MS	SP-2250
HI-225(ms)	SP-2300
InertCap 225	SPB-225
MEGA-225 (MS)	TRB-225
Optima 225	WM-225

G5 (high 3-cyanopropylphenyl polysiloxane) **G8** (90% 3-Cyanopropyl, 10% phenylmethylsilicone) **G48** (partially cross-linked cyanopolysiloxane)	
AT-Silar	MEGA-10 FAMEs
BPX70	MEGA-50
CA-2330	Quadrex 007-23
CA-2340	Rt-225
CA-2560	Rt-2330
Ciola-1301	Rt-2340
CP-Sil 88 (for FAME)	Rt-2560
DB-23	Rtx-2330
DB-Dioxin	Select FAMe
DB-FastFAME	SH-Rt-2560
Elite-23	SH-Rtx-2330
Elite-2330	Silar-10CP
Elite-2560	Silar-10C
GsBP-225MS	Silar-9CP
GsBP-23	SP-2330
GsBP-2340	SP-2340
GsBP-2560	SP-2560
GsBP-88	TC2560
HI-10	TRACE TR-FAME
HI-50	TRB-225
HP-88	TR-CN100
MEGA-10	Zebron ZB-FAME

The resolving power of near peaks in GC can be enhanced setting customised chromatographic conditions, mainly related to the mobile phase flow and the oven temperature [100]. In most of the methods developed for FAs separation, a gradient of temperature is applied during the chromatographic run, in order to speed up the separation.

At the end of the chromatographic run, FAMEs are detected by different detection systems, generally consisting in flame ionization detection (FID) or mass spectrometry (MS). The FID is a mass-sensitive and destructive detector, characterised by high robustness and high sensitivity. In this kind of detector, a flame made up by a flow of hydrogen and air 1/10 reacts with the carbon present in the organic molecules of the sample passing through the detector within the mobile flow. The reaction produces the formation of ions, whose movement is measured as the electric current between two electrodes, and generates a signal associated with each compound [101]. The response of the FID is generally stable and linear with concentration of the analytes up to seven orders of magnitude (dynamic linear range up to 107). FID detection allows FAMEs identification by comparison of their retention times with those of analytical standards analysed under the same conditions of the sample.

On the other hand, MS is a detection technique that works through the separation of molecules of a sample after their ionization, based on their characteristic m/z (mass to charge) ratio. The result obtained after the detection process is represented by the mass spectrum, a bar diagram in which the relative abundance of any ion is plotted against its m/z value. In a mass spectrum, the base peak is the most intense, and it is used as reference (as 100%) to express the abundance of other ions [102]. In combination with GC, MS is commonly employed providing substance-specific information for each separated component even when present at trace levels in a complex mixture. The sensitivity of the MS analyser is as high as, or even better than the FID [103]. For GC-MS analysis, electron ionization (EI) is the most used technique to produce ions from neutral molecules entering the mass spectrometer via the GC capillary by means of a collision of gaseous molecules of the analytes with a flow of electrons emitted by a glowing filament and the subsequent formation of a positively charged ion. All the charged species created in this process are focused, accelerated, and transmitted through electric or magnetic fields of mass analysers, whereas radicals and neutral molecules are removed by the pumping system [103]. The fragmentation pattern is strictly dependent on the structure and the chemical-physical properties (weight and internal energy) of the single molecules and on the energy of the reaction. Most of the mass spectrometers integrated in a GC-MS system operate at a fixed energy of 70 eV, which guarantees reproducible high ionization efficiency [103]. Spectra generated at 70 eV electron energy show well-reproducible fragmentation pattern independent of the instrument. Thus, EI mass spectra can be compared with those collected in mass-spectral libraries in order to tentatively recognise the compounds present in the mixture, even if the presence of homologues, isomers, or related structures can lead to error in identification in the absence of analytical standards [104].

Since MS can provide structural information for FAs identification with higher efficiency and better selectivity, allowing additional peak identification by comparison with commercially available spectral libraries, GC-MS represents the preferred analytical when analysing complex samples containing contaminants, artefacts, or co-eluting compounds [1,11,105]. Furthermore, when choosing MS as the detection method after the chromatographic separation of FAs, the signal acquisition can be performed using the selected ion monitoring (SIM) mode, selecting a single (or multiple) prominent ion(s) instead of the full mass spectrum, achieving a higher signal-to-noise ratio [105]. In this way, an appropriate adjustment of SIM acquisition parameters can be useful for accurately identifying and quantifying co-eluting FAs. Thurnhofer et al. [106] demonstrated that a GC/EI-MS-SIM method developed and employed in FAs profiling of cod liver oil was ~20- and ~10-fold more sensitive than GC/EI-MS-full scan and GC/FID, respectively, especially for the determination of minor FAs. However, one drawback of GC-MS is represented by the fact that spectrometry methods are more complex and challenging to implement and calibrate, requiring stronger knowledge and experience of the user in together with greater routine maintenance of instrumentation.

FAs quantification is performed by the integration of chromatographic peak areas given by the signal of the compound (GC-FID and full-scan MS) or selected ion (GC-MS), which are generally proportional to the absolute concentrations of individual FAMEs in the injected mixture. The chromatographic response of each FAME is not linear and may vary depending on the structure of the compound, especially the carbon chain length and the unsaturation degree. When working with FID detection, the carboxyl atom in each ester is not appreciably ionized during combustion, thus GC-FID data need to be corrected by means of empirical or theoretical factors [107,108] in order to achieve an adequate reproducibility and accuracy for quantification of most of FAs in fish tissues [93]. On the other hand, when working with GC-MS, the chromatographic response of peaks depends both on the ionization method (electron vs chemical) and the type of mass detector employed (quadrupole vs ion trap) [105,109].

Quantitative data can be expressed as relative (% of total FAs) or absolute contents (mg FA/g of sample, mg FA/g of total lipids, etc.). Quantification can be achieved adding an internal standard (IS) to the sample, a compound characterised by chemical structure as closer as possible to most of the FAMEs to be quantified. The IS cannot be present in the sample analysed and cannot co-elute with any of the FAMEs to be quantified. The IS most employed with aquatic samples are non-adecanoic acid (C19:0), heneicosanoic acid (C21:0), and tricosanoic acid (C23:0) [1].

20.6 NOVEL ANALYTICAL STRATEGIES AND TOOLS: MULTIDIMENSIONAL GC AND LIPIDOMICS APPROACH

The rapid advances in available technologies and knowledge occurred in recent times allowed lipid chemists to develop and apply alternative methods to conventional techniques for FAs analysis. The traditional method for determining FAs profile can be time-consuming and laborious, above all when working with a high number of samples. For this reason, researchers tackled to develop alternative methods in order to reduce the analytical effort but still achieving high performances. As an example, recently, Perez-Palacios et al. [110] proposed a novel method based on a direct transmethylation procedure followed by a fast GC-FID run, without any lipid extraction and purification steps, for analysing FAs in meat products, obtaining suitable results in terms of sensitivity (LOD and LOQ), precision, and accuracy. Similarly, Kenar et al. [111] proposed a methodology based on direct electron ionization-mass spectrometry (EI-MS) of FAMEs extracted by edible oils, without any GC separation. If coupled to proper chemometric techniques, the EI-MS method was demonstrated to provide a rapid, simple, and accurate tool to assess edible oils authenticity, in an untargeted approach where FAs identification was not required to correctly differentiate different samples. However, the authors concluded that if the target of the analysis is an exact determination of individual FAs, a chromatographic separation is needed.

Novel hyphenated techniques are going to be more and more employed in FAs analysis. Among them, multi-dimensional GC (MDGC) has been attracting increasing interest among researchers, being able to provide greater resolving power and enhance peak capacity and sensitivity in shorter analytical times if compared to unidimensional GC [11]. Consequently, MDGC techniques have been employed for determining FAs profile in several types of samples, including animal tissue samples and fish oils [112, 113, 114].

Further, liquid chromatography (LC) methods coupled with different detection systems have been successfully employed in both targeted and untargeted strategies applied to lipid analysis. Particularly, the ability to frame advanced hyphenated techniques based on LC separation is leading to the development of sophisticated methods allowing to save time generally required for sample preparation and analysis in conventional GC-based techniques. Several LC methods have been developed for the analysis of both derivatised and underivatised FAs. Methods comprising the derivatisation of FAs allow the determination of

FAs included in all the lipid fractions. For example, Nagumalli et al. [115] developed a novel method for the analysis of FAs in triglycerides of vegetable oils characterised by a very high sensitivity, with LOD of all considered FAs in the low fg on-column range. In the procedure of Nagumalli et al. [115], FAs were first derivatised in their DMAE-ester derivatives and then analysed by UPLC-ESI-MS/MS (positive ESI, ACQUITY BEH phenyl column), allowing an isocratic resolution of all FAs derivatives in 1.5 min chromatographic run.

On the other hand, LC methods not comprising the derivatisation steps are particularly suitable for the analysis of FFAs. For example, Lee-Okada et al. [116] developed a method for measuring FFAs by means of reverse-phase LC-ESI-MS/MS without any prior derivatisation, obtaining the semi-quantitative estimation of a broad spectrum of FFAs in the mice liver and serum.

In recent times, the attention of the scientific community is focusing on the characterisation of the molecular species in each lipid class, with special interest for the PLs fraction and high-throughput and advanced LC-MS methods are the most widely used in lipidomics studies. Actually, if GC-based methods are only suitable to depict the profile of FA chains included in lipid complexes, LC-based methods allow the analysis of the whole lipid molecules, especially when coupled with MS/MS, dramatically increasing the potential of lipid research. Lipidomics approaches aim to determine the structures of lipid molecules from MS data and to reveal any change in samples related to these compounds due to different factors under analysis in both targeted and untargeted strategies. These objectives are achievable thanks to the high potential of combining powerful techniques such as soft ionization and high-resolution mass spectrometry (both full-scan and MS/MS profiles) with LC separation [117].

Among them, UHPLC-Q-Exactive Orbitrap-MS is one of the most powerful methods widely employed for a comprehensive investigation of lipidome in food of aquatic origin. By means of this hyphenated technique, more than 1,000 lipid molecular species, belonging to different subclasses (TAGs, DAGs, FFAs, PCs, PEs, SMs, and so on), can be identified in aquatic resources by means of fragmentation and MS/MS spectra [118, 119, 120, 121, 122]. For example, Li et al. [119] and Shen et al. [122] focused their analysis on the phospholipid fraction of some seafood processing by-products (shrimp waste, codfish roes, squid gonad). Employing a UHPLC-Q-Exactive Orbitrap-MS method, the authors were able to detect several PL molecular species containing different kinds of fatty acid chains, revealing a great presence of n-3 PUFAs structured PLs, recognised as very effective in improving human health, in the by-products analysed.

Furthermore, the outcomes obtained in lipidomics studies could be very useful for disclosing the proportion of the different lipid classes in the matrices analysed and to characterise the identity of FAs encompassed in complex lipid molecules, providing useful information about their distributionand function. Such an example, Cui et al. [118] and He et al. [120] analysed the whole lipidome of golden threadfin bream (Nemipterus virgatus) and in round scad (Decapterus maruadsi) by UHPLC-Q-Exactive-Orbitrap MS, finding that DHA tended to concentrate more on PLs than TAGs and other lipid classes in such samples. Moreover, the authors revealed that DHA was preferentially bonded to the sn-2 position of TAGs. This outcome acquired great importance since it has been demonstrated that FAs are better utilised and less prone to oxidation when located on specific position of TAGs (mainly, in the sn-2 position for PUFAs and external positions for MCFAs) [123,124].

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