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Design, development and characterization of DHA-based emulsion for Nutrilipidomics strategies

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1. Fatty acid-based membrane lipidomics

1.1 Plasma membrane

The cell membrane is crucial for the organization and functioning of every living cell. It constitutes the physical and semi-permeable barrier separating the intracellular from the extracellular environments, thus circumscribing the cellular content [1]. The plasma membrane is involved in every aspect of cellular fate, from cell division to programmed cell death [2] [3] [4]. Besides its structural role, it is involved in the regulation of transmembrane transport of small molecules either through passive or active mechanisms. Cell membrane also participates in various cellular processes such as cell adhesion, recognition and signaling [1] [5].

Structure

The plasma membrane is a dynamic, complex structure of lipids and proteins in a fluid state, organized in the well-known mosaic model, according to which most of its molecules are able to move about in the plane of the membrane [6]. The most abundant membrane lipid species are the amphipathic glycerol-based phospholipids, which spontaneously form the lipid bilayer due to the hydrophobic effect [7]. Therefore, the major core of a biological membrane is self-organized, with the hydrophobic tails being segregated from water and facing each other, while the polar headgroups form an ionic surface interacting with water (Figure 1). Cholesterol is another major lipid component of the plasma membrane, which is embedded in the phospholipid bilayer and contributes to its fluidity regulation [8]. In the lipid-cholesterol assembly proteins have their place, such as trans-membrane, anchored and peripheral proteins [8] [9], and the membrane phospholipids have an important role for the specific protein activities. This subject will not be treated in this thesis.

In addition, the plasma membranes may also contain glycolipids, glycoproteins, glycerophospholipids, sphingolipids and plasmalogenis. The relative distribution of the phospholipids varies within the different cell types and tissues. However, PC is the most abundant (>50%) phospholipid in eukaryotic membranes.

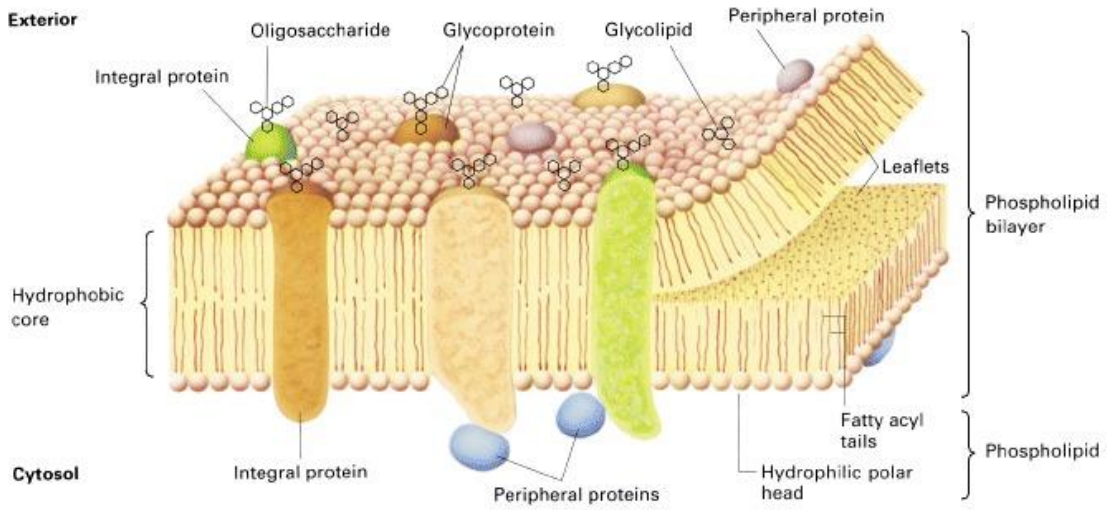


FIG 1: Schematic diagram of the structure of a typical biological membrane. The phospholipid bilayer, the main matrix of all cellular membranes, is made of two phospholipid leaflets whose fatty acyl tails form the hydrophobic core of the bilayer; their polar, hydrophilic head groups face the aqueous cytoplasm and extracellular environment. Integral proteins are embedded in the bilayer and peripheral ones are mainly associated with the membrane by specific protein-protein interactions. Oligosaccharides bind to membrane proteins and lipids, forming glycoproteins and glycolipids, respectively.

As mentioned above, the fundamental matrix of the plasma membrane is the lipid bilayer, which is formed by phospholipids. Every phospholipid molecule is made of a hydrophilic head and a hydrophobic tail, as shown in Figure 2.

The phospholipids are asymmetrically distributed between the two leaflets of the membrane bilayer. The outer layer contains mainly PC and SM, whereas the inner leaflet consists mainly of PE and PS.

The asymmetric lipid distribution between the two leaflets contributes to curvature stress in biologyical membranes, which is useful for the membrane budding, fission and fusion [9] as well as the conformation of membrane proteins and modulation of their function. Considering the variety of phospholipids' headgroups and their combination with several different fatty acids, the content of individual phospholipid species can be relatively diverse. This results in increasing membrane's flexibility and may be needed for the numerous processes in which phospholipids have been reported to be involved [10].

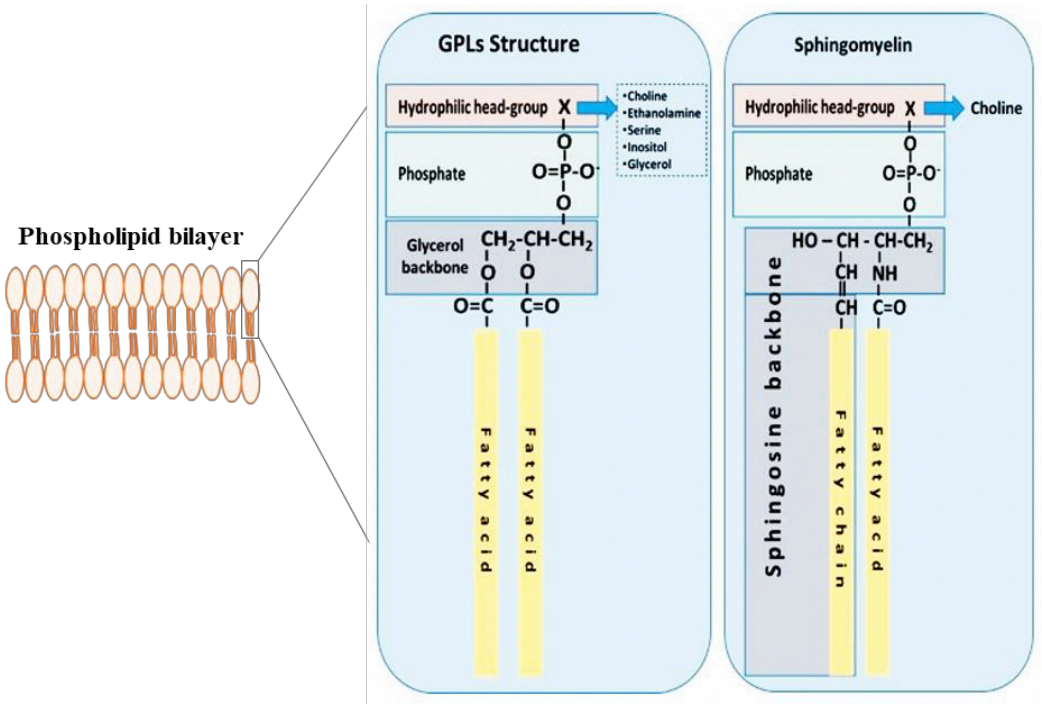


FIG 2: Common structures of glycerophospholipids (GPLs) with a glycerol backbone and sphingomyelin as a representative of a sphingophospholipid (SPLs). Modified from Lordan R *et al.*, 2017**,** *Molecules*, MDPI.

Physicochemical properties

The plasma membrane is characterized by various physicochemical properties, such as the membrane as the fluidity. However, it is reported that the physical properties of any lipid mixture are a collective property determined by the single lipid components [11]. The plasma membrane is constituted of a large variety of lipid molecules, each of them having different physical properties. As a result, every lipid moiety contributes collectively to the final properties of the membrane assembly.

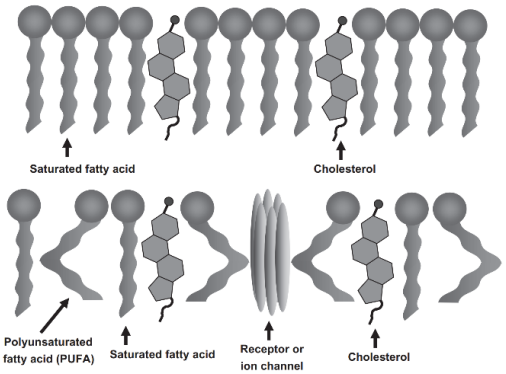


FIG 3: The structure of fatty acid chain influences the membrane fluidity. Saturated fatty acids form a more rigid and less fluid lipid bilayer due to the tight packing of the straight chains. The double bonds of unsaturated fatty acids create a bend in the fatty acid tail, thus leading to increased membrane fluidity. Modified from Jich et al. (2010) [12].

The physical and chemical properties of the membrane influence several cellular processes, thus suggesting that the lipids are not only structural components, but they also have a dynamic role in cell function [13] [14]. A key factor affecting the physicochemical properties of the membrane is the type of fatty acid moieties that are present to the phospholipid molecules [15]. The fluidity of the hydrophobic domains of the phospholipids is a function of the fatty acid chain structure and temperature [16], as depicted in Figure 3. At a given temperature the fluidity of the hydrocarbon core of the membrane bilayer increases with higher content of unsaturated alkyl chain or with smaller alkyl chain length. Most phospholipid molecules have one *cis*-unsaturated fatty acyl chain, which enables them fluid at room temperature. Higher temperature leads to increased mobility of the fatty acid chains, which in turn increases the fluidity and the space occupied by the hydrophobic domain of lipids.

1.2 Membrane fatty acids

As mentioned above the major building block of membrane assembly is the phospholipid molecule whose hydrophobic tail contains usually two long fatty acid chains. The fatty acids (FA) are aliphatic chains with a carboxylic acid group (-COOH) at the end of the chain. Fatty acids naturally occurring in phospholipids commonly have a chain of 14 to 24 carbons (usually unbranched and even-numbered), which may be saturated or unsaturated.

Odd-numbered fatty acids are most frequent in bacteria and lower plants or animals [17]. In eukaryotes, the fatty acid chains are esterified to the positions C1 and C2 of the L- glycerol, as depicted in Figure 2. The acyl chain can vary in length and degree of unsaturation, thus affecting the properties of each moiety.

In general, fatty acids can be divided according to a) the chain length into short (C2-8), medium (C8-10) and long (>C12), b) the degree of saturation into saturated and unsaturated, c) the biological value into essential and non-essential and d) the chain structure into aliphatic, branched and cyclic. Membrane fatty acids, which consist of the hydrophobic domain of phospholipids, contain long aliphatic alkyl chains, with an even number of carbon atoms, typically atom ranging between 14 and 24. For this reason, further description of fatty acid classification in this chapter will concern only their saturation degree and their biological significance.

The hydrocarbon chain of membrane fatty acids can vary in the number of double bonds and thus be classified into saturated or unsaturated. The differences between saturated and unsaturated fatty

acids, as well as the variation in geometry of unsaturated FA, determine the properties of membrane structure, thus influencing its microdomain organization and altering numerous cellular processes [18].

Saturated fatty acids

Saturated fatty acids (SFA) do not contain any double bond along the alkyl chain. The term 'saturated' is used because all the carbon atoms contain as many hydrogen atoms as possible. The general formula of SFA is $CH_3(CH_2)_nCOOH$. The lack of any double bonds or other functional groups enable these fatty acids to be nearly chemically inert and thus subject to drastic chemical conditions, such as temperature and oxidation. In Figure 4, the structures of two representative members of the SFA family are depicted.

Palmitic (16:0) and stearic (18:0) acid are the two most commonly occurring SFA. The long SFA are characterized by high melting points, which means that they are in the solid state at room temperature. This physicochemical behavior is a consequence of their molecular structure (straight shape chains) that contributes to the high packing of SFA in phospholipid bilayer.

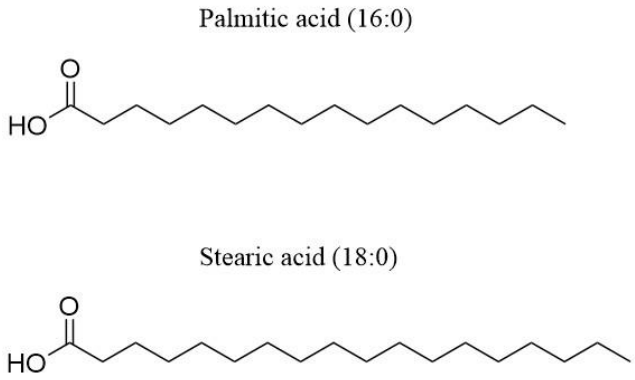


FIG 4: Molecular structure of representative members of saturated fatty acids (SFA) family. Palmitic (16:0) and stearic (18:0) acid are characterized by straight chains that differ in length by two carbon atoms.

Unsaturated fatty acids

The hydrocarbon chain of unsaturated fatty acids (UFA) may contain one or more double bonds. Unsaturated fatty acids exhibit the positional and geometrical isomerism at the double bonds, which are characterized by *cis*-configuration in the majority of naturally occurring UFA. According to the number of double bonds, the unsaturated fatty acids can be further divided into mono- and polyunsaturated FA.

- Monounsaturated fatty acids (MUFA)

  Monounsaturated FA contain only one double bond along the alkyl chain. The general formula of MUFA is $CH_3(CH_2)_xCH=CH(CH_2)_yCOOH$. The position of the unique double bond can vary a lot. The presence of the double bond increases the FA fluidity since a double bond in the *cis* configuration provokes a bend in the alkyl chain, as shown in Figure 1.5. This bending leads to a total spatial width of 0.72 nm for a *cis*-MUFA compared to the 0.32 nm one that characterizes the saturated structures.

Unlike the SFA that have the tendency to pack in a membrane structure, MUFA cause a higher molecular disorder due to their three-dimensional shape. Therefore, their presence in membrane assembly influences its fluidity and permeability [18]. MUFA have a lower melting temperature than SFA and they are in liquid state at normal temperature and semisolid or solid when refrigerated. Amongst the most common MUFA members are the palmitoleic acid (16:1,cis-Δ9), oleic acid (18:1,cis-Δ9) and vaccenic acid (18:1,cis-Δ11).

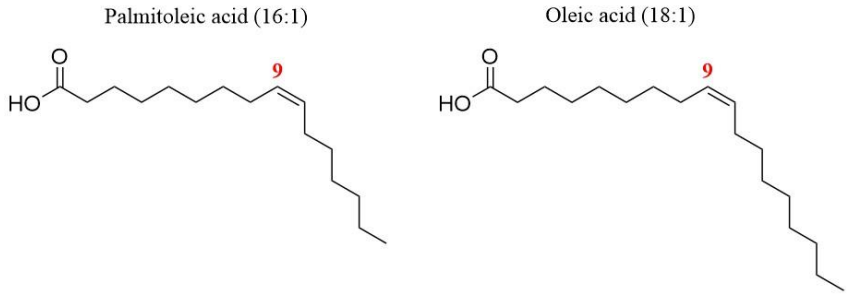


FIG 5: Molecular structure of representative members of monounsaturated fatty acids (MUFA) family. The formation of a double bond in Δ9 position creates a bend to the chain of palmitoleic (16:1-c9) and oleic (18:1-c9) acid.

- Polyunsaturated fatty acids (PUFA)

  Polyunsaturated FA contain two or more *cis* double bonds, which are usually separated from each other by a single methylene group (methylene-interrupted unsaturation) and have the general formula -C-C=C-C-C=C-. PUFA, as unsaturated fatty acids, have a more extended shape than SFA due to the presence of double bonds that increase the bending of the hydrocarbon chains. Therefore, PUFA have significantly lower melting points compared to other FA families, as presented in Table 1.1. PUFAs are important structural components and contribute to membrane fluidity and selective permeability [19]. The higher the degree of unsaturation in FA (more double bonds), the more susceptible they are to lipid peroxidation, whereas UFA can be protected from lipid peroxidation by antioxidants [20] [21]. Polyunsaturated fatty acids are usually divided into omega-6 (ω-6) and omega-3 (ω-3)

series, based on the distance between the final methyl group and the closest double bond in the chain. Thus, starting the numbering from the omega carbon, the first double bond is at the position C6 and C3 for the ω6 and ω3 family, respectively. Figure 6 shows the molecular structure of representative members of each PUFA family.

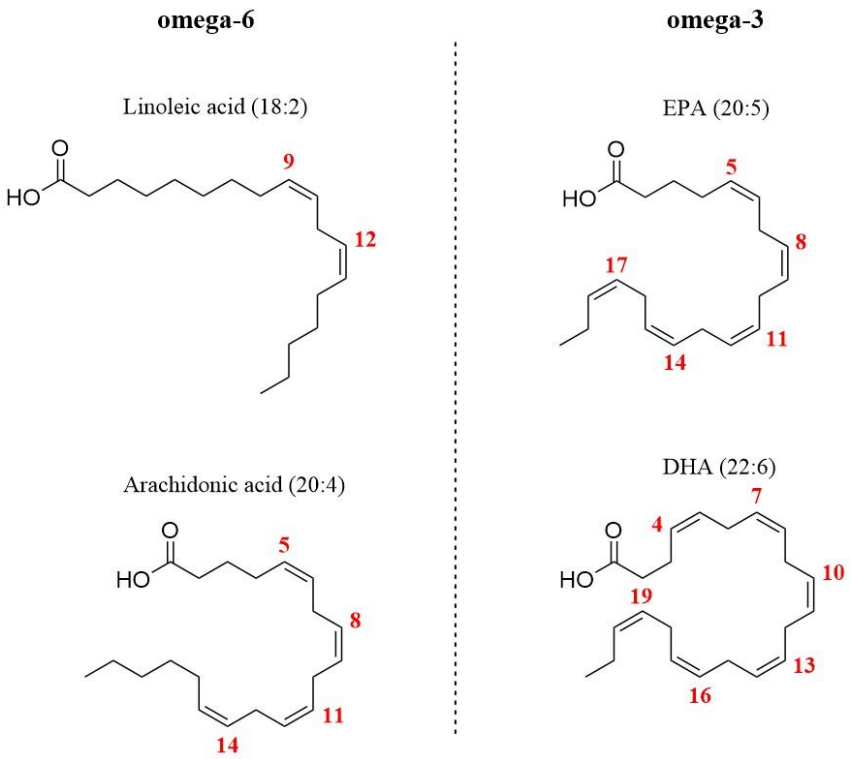


FIG 6: Molecular structure of representative members of polyunsaturated fatty acids (PUFA) family. PUFA have an extended shape due to the multiple double bonds and are generally divided into omega-6 and omega-3, based on the distance between the final methyl group and the closest double bond in the chain.

Geometrical isomerization

The double bond in unsaturated fatty acids can be either in *cis* or *trans* configuration (*Z*- or *E*-, respectively, according to IUPAC notation). In the *cis* configuration the two alkyl groups (R1 and R2) are on the same side of the double bond, where in *trans* geometry they are on the opposite sides (Figure 6). In eukaryotes, the double bond of unsaturated fatty acids has prevalently *cis* geometry. During fatty acid biosynthesis, the insertion of *cis* double bonds is catalyzed by desaturases, enzymes which act in regioselective and stereospecific way [22]. The *trans* geometry is not naturally present since these eukaryotic enzymes are not capable to form *trans* double bonds or catalyze their *cis-trans*

isomerization. The loss of the natural *cis* geometry changes dramatically the molecular shape of the fatty acid chain, thus affecting several membrane properties, such as the diameter, fluidity and permeability [23] [24]. For instance, elaidic acid (9*trans*-18:1), which is the geometrical isomer of oleic acid (9*cis*-18:1), is characterized by linear alkyl chain instead of the typical bending structure of MUFA, as shown in Figure 7.

Trans fatty acids (TFA) can originate by the chemical manipulation of natural oils, such as the deodorization or partially hydrogenation [25] [26]. Some TFA occur in the dairy products, milk and meat, specifically due to the microbial biohydrogenation that takes place in the ruminant stomach [27]. Contrary to eukaryotes, some Gram-negative bacteria have the ability of endogenous *cis–trans* isomerization as a response to environmental stress conditions [28]. In eukaryotic cells, the conversion of the natural *cis* fatty acids to their *trans* isomers may occur endogenously under stress condition and involves a thiyl radical catalytic mechanism [29] [30] [31].

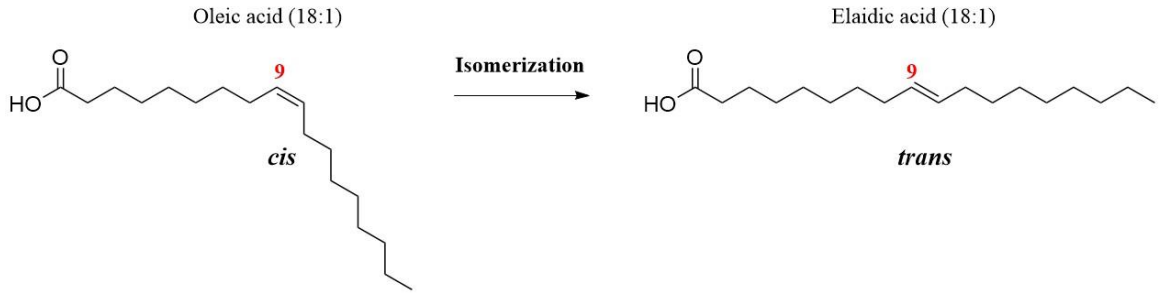


FIG 7: Example of geometrical *cis-trans* isomerization in fatty acids. The configuration of the double bond influences the shape of the alkyl chain; *cis* geometry produces a kink in the molecule, while *trans* geometry favors a linear carbon chain.

Biosynthetic vs essential fatty acids

Mammals can synthesize saturated and monounsaturated fatty acids, but they are unable to synthesize FA containing more than one double bond (PUFA). Indeed, they lack the enzyme system that is responsible for the introduction of a double bond in a monounsaturated alkyl chain. Consequently, the fatty acids not being synthesized by the organism should be supplied through diet and are known as essential or semi-essential FA.

De novo biosynthesis

In adult humans, the *de novo* synthesis of fatty acids is taking place mainly in the liver, adipose tissue, and lactating breast. The fatty acids that contain up to 16 carbon atoms are synthesized by the fatty

acid synthase (FAS). FAS is a cytoplasmic enzyme that is composed of two similar subunits (~250 kDa each) and acts as a multifunctional complex. It is characterized by seven different enzymatic activities within two catalytic centers. Fatty acid synthesis by FAS is initiated by the condensation of an acetyl-CoA and malonyl-CoA molecule, while NADPH serves as the reductant in this process. The addition of a two carbon-unit from malonyl-CoA is repeated seven times in a cyclic manner, thus leading eventually to the production of the saturated C16 fatty acid (palmitic acid) [32] [33] [34]. Due to the mechanism of their *de novo* biosynthesis, most of the natural FA have an even number of carbon atoms. The palmitic acid is the starting point for the biosynthesis of other fatty acids, which is catalyzed by a set of microsomal enzymes generating modified alkyl chains; elongated or desaturated ones (see Figure 7). The fatty acids produced by FAS, as well as those originated from the diet, can be further elongated into very-long chain fatty acids (VLCFA) containing more than 18 carbon atoms. The overall elongation reaction takes place mainly in the endoplasmic reticulum (ER) by four membrane-bound enzymes. The enzymatic steps involved in this process are similar to the synthesis of palmitate since malonyl-CoA and NADPH are respectively used as an intermediate and reductant. The proteins performing the successive steps of VLCFA extension are individual molecules, which could be physically associated, contrary to the FAS multi-enzyme complex [35] [36]. Three of the four enzymatic activities in VLCFA elongation are localized to the cytoplasmic side of ER membranes, while the enzyme performing the third step is suggested to be embedded in the membrane [37]. In humans, seven enzymes (ELOVL 1– 7) have been identified with the ability to elongate the >16C fatty acid chains into VLCFA [38]. Despite the capability of all the seven elongation enzymes to catalyze the condensation reaction in the elongation cycle, they are characterized by differential substrate specificity and tissue distribution [39]. In addition, palmitate and other long FA can be further processed by acyl-CoA desaturases that can modify the structure and properties of long-chain fatty acids by introducing a double bond at a specific position on the acyl chain [40]. Mammalian cells express three different desaturases that are generally divided into two distinct families: stearoyl-CoA desaturases (SCDs) [41] and fatty acid desaturases (FADS) [42]. Human desaturases catalyze the introduction of a double bond at specific positions (Δ9, Δ6 and Δ5) into the saturated fatty acyl-CoA chain. SCDs, also known as Δ9 desaturases, catalyze the insertion of a single double bond at the carbon C9 (counting from the carboxylic acid group). The oleic and palmitoleic acids are the main products of SCDs synthesized by the desaturation of the SFA stearic and palmitic, respectively. In humans, two SCD isoforms (SCD1 and SCD5) have been identified, with the SCD1 being the most commonly expressed among tissues [43]. FADS1 and FADS2, which respectively have Δ5- and Δ6-desaturase activities are mainly involved in the PUFA biosynthetic pathways, as described later in this paragraph. However, FADS2 can potentially also act on palmitic

forming the sapienic acid (*cis*-6 hexadecenoic), which is reported to be the most abundant fatty acid in human sebum [44]. The formed MUFA may undergo further elongation by the previously mentioned elongation system. For example, palmitoleic (C16-Δ9) can be converted to the *cis*-vaccenic (C18-Δ11) and oleic (C18-Δ9) to gondoic acid (C20-Δ11). Consequently, this chain extension 'shifts' the position of the double bond by two carbon atoms since the elongation occurs in the carboxyl terminus. Finally, it is worth mentioning that the intracellular fatty acid pool is enriched not only by the endogenously synthetized FA but also by those obtained through the dietary habits of everyone.

Essential fatty acids

Despite their ability to synthesize saturated and monounsaturated fatty acids, animals are not capable to produce *de novo* polyunsaturated FA because they lack the required enzyme system that further desaturases oleic acid (18:1-Δ9) into linoleic acid (LA, 18:2-ω6) and α- linolenic acid (ALA, 18:3-ω3) [45]. Therefore, these two PUFA moieties are considered as dietary essential fatty acids as they cannot be endogenously synthesized. However, they themselves or their metabolic derivatives play an important role in human health and development by being involved in numerous biological functions [46] [47]. For instance, arachidonic acid (20:4-ω6), a metabolic derivative of linoleic acid, is the precursor molecule for the synthesis of prostaglandins by the cyclooxygenase enzyme system and leukotrienes by the lipoxygenase pathway in leucocytes [48]. The other members of PUFA family can be provided either through the dietary intake or synthesized from the nutritionally essential fatty acids (LA and ALA). LA and ALA are the starting points of PUFA biosynthesis in humans and can be further modified by the activity of desaturases and elongases, as depicted in Figure 8 [49]. As previously mentioned, PUFA can be divided into the omega-6 and omega-3 families, based on the position of the double bond closest to the methyl end of the alkyl chain. The precursors of these families (LA for ω-6 and ALA for ω-3) can be transformed to more highly unsaturated FA by a series of common elongation and desaturation reactions in ER [50]. FADS1, FADS2 are the key desaturases in PUFA biosynthesis. For example, arachidonic acid (ARA), a long-chain ω-6 PUFA, is synthesized from LA, through the following successive reaction steps: the addition of a double bond by Δ6-desaturase to form γ-linolenic acid (GLA, 18:3-ω6), the elongation of GLA to form dihomo-γ-linolenic acid (DGLA, 20:3-ω6) and finally the addition of another double bond by Δ5-desaturase to form eicosatetraenoic acid (ARA, 20:4-ω6) [51]. Similar desaturation and elongation steps are utilized for the formation of eicosapentaenoic acid (EPA, 20:5-ω3), a member of omega-3 family. In higher eukaryotes for the synthesis of DHA, a downstream derivative of EPA, the latter is elongated to DPA-ω3 which is further elongated into tetracosapentaenoic acid (TPA, C24:5-ω3) and then TPA

is desaturated to form ω3-tetracosahexaenoic acid (THA, C24:6-ω3) by a Δ6-desaturase. Finally, the THA undergoes beta-oxidation in peroxisomes to form the DHA-ω3 [52] [53]. On the contrary, lower eukaryotes are able to elongate the EPA-ω3 into DPA-ω3 and then desaturate the latter into DHA-ω3 by the Δ4-desaturase, an enzyme that is not present in mammals [54]. PUFA family members, such as ARA and DHA are considered as semi-essential fatty acids, since they can be synthesized endogenously by their precursors (LA or ALA). Semi- essential FA turn into essential ones in case their precursors are missing from the diet. Interestingly, *in vitro* studies in glioma cells have shown that the occurrence of double bonds with *trans* configuration in PUFA influences the metabolic fate of the latter by affecting the processes of desaturation and elongation in the fatty acid chain [55] [56].

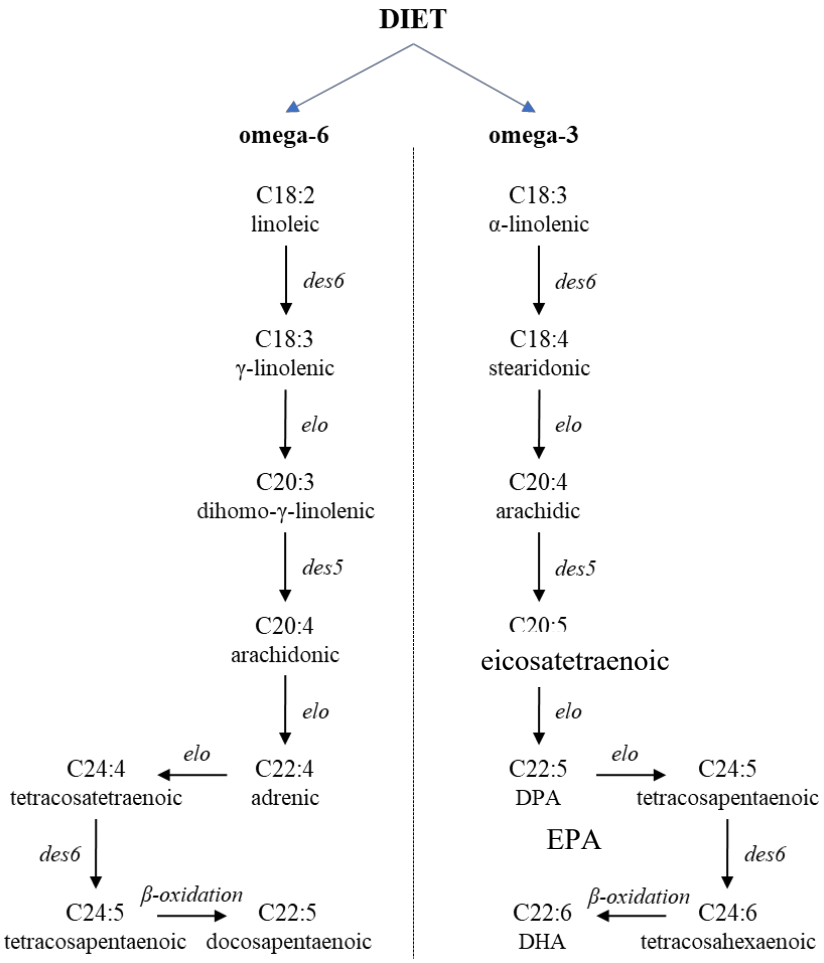


FIG 8: PUFA biosynthesis in mammals. Linoleic (LA) and α-linolenic (ALA) acids are respectively the precursors of omega-6 and omega-3 family. LA and ALA are further converted to long chain fatty acids using a series of desaturation and elongation reactions in the endoplasmic reticulum. The final products of omega-6 and omega-3 biosynthetic pathways, DPA-ω6 and DHA- ω3, respectively, are formed by β-oxidation in peroxisomes. Modified from Lauritzen et al. (2001) [57]. *elo*: elongase, *des5/6*: Δ5-/6-desaturase.

2. Lipidomics for lipid biomarker discovery

Variations in the composition of human body lipids can be monitored through lipidomics, which represents a powerful diagnostic tool to assess the quantity and quality of FA constituents and to monitor the remodeling of membrane FA composition associated with various physiological and pathological conditions [58].

Lipidomics aims at understanding membrane lipid role in the functioning and regulation of cellular behavior. Indeed, it is possible to detect lipid variations occurring in health and disease situations, in order to gain knowledge on their connections with complex mechanisms of cellular homeostasis and signaling. The "dynamic" perspective offered by lipidomics allows the possibility of deepening the knowledge about the individual heritage and lipid transformation and of relating lipid type and quantity with health conditions. It is now possible to identify, for each tissue, the normality ranges for membrane FA composition in standard health conditions.

It is worth mentioning that lipidomics is often described and considered as a part of metabolomics. However, this collocation only takes into consideration the study of lipid structural changes caused by metabolic pathways, whereas lipids have an important constitutive role as main components of membranes connected with the whole cell regulation and signaling. As described further, membrane lipidome is carefully evaluated in functional lipidomics to gather relevant information of cell homeostasis (or allostasis) and adaptation. Phospholipid remodeling is part of an important short-term adaptation response; fatty acids detached from membrane phospholipids propagate the effect of environmental and metabolic stimuli that arrive to the cell and drive gene expression. Based on this scenario, lipidomics can relate to genomics and proteomics, all of which constitute the family of system biology [59].

Membrane lipidomics includes the comprehensive and quantitative description of membrane lipid constituents. Membrane structure is very well known to be a double layer of phospholipids which have an amphiphilic character due to the coexistence of the two hydrophobic fatty acid chains and a polar group. In the last two decades the roles of the cell membrane have been refocused and addressed for most of the cellular activities, and the definition of ‘metabolic pacemaker’ well describes the network of activities and signaling regulated by the membrane asset [60].

To date, lipidomic analytical strategies are applied to a wide variety of biological samples, such as blood, plasma, serum, cerebrospinal fluid, urine and biological tissue derived from animal models or

clinical patients. In particular, red blood cells (RBCs) can be ideal reporters for evaluating individual values and possible deviations connected with intrinsic or extrinsic metabolic reasons (e.g. inflammation, stress and dysfunctional diet). Indeed, unlike the analyses, which are carried out on plasma and give information about FA dietary intakes of a few weeks before withdrawal, RBC lipidomic analyses account for more stable information obtained from metabolic transformations together with stabilized dietary contributions.

In this view, the fatty acid based lipidomics approach could contribute to underline alterations in the lipid metabolism or in both physiological and pathological conditions, information that can be used as guidance for personalized medicine, dietary and nutraceutical intervention, as well as in biomarker discovery. Indeed, lipidomics has been successfully applied to discover biomarkers for a variety of diseases, such as metabolic and cardiovascular disease.

Moreover, lipidomics can play a key role in mechanistic studies, risk prediction, and therapeutic monitoring for such pathologies in which aberrant lipid metabolism occur, such as diabetes and obesity. Since lipids play important roles in cell growth and metabolism, which are essential for rapidly proliferating cancer cells, lipidomics has been used to report changes in lipid metabolism and homeostasis undergone by cancer cells [61].

Lipidomic analysis also clarified the role of some lipid-derived bioactive molecules that are involved in ophthalmological processes related with eye disease [62].

Lipidomics has been used as well to understand diet-induced changes in the structure, composition, and function of cellular lipids. Additionally, lipidomics might be useful for evaluating chronic effects associated with the dietary intake of specific components and for providing nutritional advice and lifestyle corrections.

The goal is to integrate the lipidomic knowledge with therapeutical, nutritional and nutraceutical aspects, and include lipids as important elements for prevention strategies and integrated medicine.

2.1 Autism Spectrum Disorder (ASD)

ASD is a neurodevelopmental disorder characterized by impairment of social interaction and deficiencies of verbal and nonverbal communication, which causes interest deficits and repetitive behaviors [63].

This condition is characterized by behavioral alterations that can be classified in three main areas and be employed as diagnostic parameters [64] [65]:

- Social Impairment: it refers to the inability of establishing social and/or emotional relationships with other people. Usually ASD children show little interest in other children or adults, do not react when called by name and become aggressive in noisy and unusual environments.
- Communication: it refers to the inability of expressing ideas and feelings. Usually, delay or total lack of language are seen. If language is present, it is often abnormal and consisting of repeated sounds or single words which cannot be organized in sentences. They also display problems in understanding conversations and concepts.
- Rigidity and perseveration: it refers to a constant dedication to one or more types of interest with exaggerated reactions to specific topics or events (i.e. ability to memorize with no efforts name lists, historical events, sportive charts or to solve mathematic problems). The activities of children affected by ASD are usually characterized by repetitive and stereotyped behaviors. They repeatedly perform purposeless motor movements and they show persistent attachment to objects or toys that reflets anxiety and resistance to changes.

  Finally, other typical autistic behaviors are abrupt mood swings, hyperactivity or apathy, self-aggressive behaviors such as hand biting or hair grabbing, phobias, sleep disturbances and eating disorders.

  Since the variety of symptoms makes it difficult to provide a consistent clinical description, within the ASD definition are included several pathologies or syndromes characterized by the same behaviors, although at different degrees of intensity. The severity of the pathological disorder and the appearance of the symptoms are very variable, and this fact makes autism diagnosis and personalized interventions very difficult.

The incidence of the disorder in 2007 was reported to be of 6.6 cases per thousand children [66], but the US Centers for Disease Control and Prevention have recently reported the prevalence of ASD in 14.7 per thousand of 8-years-old children. This suggests that the incidence of ASD has dramatically increased. There is a marked difference in prevalence among males and females: for every ASD female with autism there are three to four males with the same disorder [67].

Parents usually notice the first signs within the baby's first two years of life and reliable diagnosis can often be made within the thirty months of life. For the diagnosis the Childhood Autism Rating Scale (CARS), a widely recognized investigation tool for ASD, can be employed [68].

CARS consists on the evaluation of children referring to a list of 15 items related to the main behavioral areas. For each point, a seven-degree scale can be used to indicate how much the subject behavior deviates from the norm relating to his age, assessing 1 as the normal behavior and 7 as the most abnormal one. The final CARS score allows to distinguish mild to medium ASD from the more severe cases.

2.2 Lipidomics for ASD biomarkers

ASD is a multifactorial disorder which is thought to have correlations with genetic, epigenetic, environmental and immunological factors, with oxidative stress as linking mechanism [69] [70] [71] [72] [73].

The brain is highly vulnerable to oxidative stress; the reason can be found in the limited antioxidant ability of this organ and in its high requirement of energy, especially in the form of lipids and iron. Therefore, neuron functionality is the first one to be compromised in the presence of ROS and shortage of antioxidant.

Children have a natural deficit in detoxification capacity and associated lower levels of glutathione compared to adults [74]. Additionally, environmental factors that induce oxidative stress were found to accumulate in the placenta and to be found in higher concentration in infants [75].

Taken together, these studies suggest that children are highly vulnerable to oxidative stress and that certain conditions could compromise normal neurodevelopment and increase the risk of neurodevelopmental disorders such as autism.

Plasmatic levels of lipid peroxidation indicators were found to be increased in ASD children compared to developmentally normal, aged-matched children [76]. Several studies have suggested alterations in the antioxidant enzymes. Children with ASD showed reduced activity of glutathione peroxidase in plasma and erythrocytes, lower levels of total glutathione, and decreased catalase and

SOD activity in erythrocytes [77] [78]. Additionally, other antioxidant proteins, transferrin and ceruloplasmin, were found to be reduced in autistic children. Being trasferrin an iron-binding protein

and ceruloplasmin a copper-binding protein, abnormalities in the iron and copper metabolism have been suggested to have, together with oxidative stress, a pathological role in autism [79] [80].

In a study from the ISOF group, directed by Dr. Ferreri, membrane alterations in autistic children erythrocyte were described. In particular, an increase in MUFAs, a decrease EPA and DHA, and a consequently increased ω-6/ω- 3 ratio that was associated with a reduction of the erythrocyte membrane fluidity, was observed. Increased levels of thiobarbituric acid reactive substances, urinary isoprostane, and hexanoyl-lysine, together with a significant reduction of Na^+/K^+-ATPase activity were also reported [76].

There are increasing evidences of abnormalities in membrane lipid metabolism and in immune and inflammatory responses in autism [81] [82]. Other studies focused on the role of fatty acid deficiencies or imbalances in the appearance of neurodevelopmental disorders such as attention-deficit/hyperactivity disorder, dyslexia, dyspraxia, and autistic spectrum disorders [83] [84]. Scientific evidences have been collected on the deficiency of EFAs, possibly correlated with overactive/over- expressed PLA2 enzymes [85] [86].

Plasma methionine and S-adenosylmethionine to S-adenosylhomocysteine ratio, an indicator of methylation capacity, were significantly decreased in ASD. In addition, plasma levels of cysteine, glutathione, and the ratio of reduced to oxidized glutathione, an indication of antioxidant capacity and redox homeostasis, were significantly decreased, suggesting that impairment in the methylation ability might occur in autism [78].

In addition to high levels of oxidative stress, a state of neuroinflammation with activation of the microglia and high levels of proinflammatory cytokines have been found to play a key role in the development of histopathological alterations [87]. Recently, the possible role of trans lipids in this disease has been discussed [88].

The efficacy of ω-3 fatty acid supplementation to children with autism has been investigated in several studies [89]. ω-3 supplements were found to reduce hyperactivity and stereotypies in children with ADS after six-week treatment [90]; caregivers reported improvements in cognitive and motor skills, concentration, sleep quality and sociability as well as reductions in irritability, hyperactivity and aggression in children supplemented with ω-3-rich oils [85].

In a recent study from the ISOF group was investigate the erythrocyte plasma membrane of ASD children. The interest in RBC membrane fatty acid composition derives from the fact that mature

RBCs have a stable and representative composition that reflects the general state of the organism, connected to an in vivo exchange with tissues and lipoproteins that occurs during normal cellular metabolism.

The results showed that the most abundant SFA is palmitic acid (16:0), followed by stearic acid (18:0). Among the MUFAs, the most abundant fatty acid is oleic acid (9c, 18:1) while the most abundant PUFAs correspond to linoleic (LA, 9c,12c, 18:2) and arachidonic (ARA, 20:4 ω-6) acids. Lipidomic analyses evidenced a significant DHA decrease in ASD subjects (p value = 0.0424), as already reported for a previously studied cohort [16,60]. Using ANOVA test to compare the two groups, the DHA decrease in ASD was found to be significant (p value = 0.0344). This decrease was not attributable to dietary differences between the two groups, since food questionnaire reported fish consuption for both groups [91, 92].

Taken together, these evidences support the idea of lipid membrane homeostasis as a therapeutic target in ASD.

3. Understanding membrane fatty acids change in other conditions

The last two decades have witnessed a strong interest in cell membrane phospholipids and fatty acid modulatory effects that are involved in the adaptability of living organisms. In particular, fatty acids have crucial roles in biophysical, biochemical and signaling processes that act as sensing mechanisms and stimuli transduction, thus participating in epigenetic control pathways.

Fatty acids, as structural components of membranes and inflammation/anti-inflammatory mediators, have well-known protective and regulatory effects. They are studied as biomarkers of pathological conditions, as well as saturated and unsaturated hydrophobic moieties in membrane phospholipids that contribute to homeostasis and physiological functions. Lifestyle, nutrition, metabolism and stress-with an excess of radical and oxidative processes-cause fatty acid changes.

3.1 The case of obesity

Interventions to control obesity have typically consisted of combined strategies including diet, exercise, and behavior therapy. Despite efforts by governments, the food industry, and the science community, obesity and overweight rates keep increasing in both child and adult populations worldwide, demonstrating a need for personalized strategies that guarantee the success of interventions in treating obesity.

The focus on early clinical markers for overweight/obesity onset is, nowadays, a clear research target. A new trend to focus on fats, and specifically the quality of dietary lipids, is crucial for the prevention and treatment of obesity. In this sense, a strong contribution from the molecular approach developed in the last two decades, characterizing fat accumulation, highlights different kinds of signaling occurring in this disease and leading to comorbidities. Since fat accumulation is strictly connected with the quality and quantity of fatty acids (FAs) in human tissues, the lipidomic approach was found to have a key role in describing the scenario of molecular signaling, providing crucial information on the various phases of weight increase, from overweight to obesity. Indeed, membrane fatty acid-based lipidomics has reached a high technology readiness level, developing simple (i.e., inexpensive and high-throughput) and robust analytics of high resolving power, as demonstrated by several applications to diseases.

It is worth recalling that to measure the lipid composition of blood, different blood compartments have been targeted. Plasma or serum FA levels have been widely analyzed because they reflect short-term dietary fat intake. However, analysis of lipid compositions from mature red blood cell (RBC) membranes offer an advantage over analysis of plasma because these cells last on average 120 days in the blood compared to 3 weeks for platelet or plasma lipids, reflecting better long-term dietary FA intake and tissue conditions. Apart from this, RBCs maintain a more stable FA composition compared to plasma FA levels.

The aim of the study of lipidomic profiles in the frame of the collaboration projects with the group of Dr. Ferreri at CNR Bologna and the group of Dr. I. Tueros at AZTI, Bilbao was to determine the fatty acid (FA) profile of red blood cell (RBC) membranes as a comprehensive biomarker of children's obesity metabolism, together with the evaluation of their dietary intake [93]. An observational study was carried out on 209 children (107 healthy controls, 41 who were overweight and 61 with obesity) between 6 and 16 years of age. Mature RBC membrane phospholipids were analyzed for FA composition. Dietary habits were evaluated using validated food frequency

questionnaires (FFQ) and the Mediterranean Diet Quality Index for children (KIDMED) test. Compared to children with normal weight, children with obesity showed an inflammatory profile in mature RBC FAs, evidenced by higher levels of ω-6 polyunsaturated FAs (mainly arachidonic acid, $p < 0.001$). Children who were overweight or obese presented lower levels of monounsaturated FA (MUFA) compared to children with normal weight ($p = 0.001$ and $p = 0.03$, respectively), resulting in an increased saturated fatty acid (SFA)/MUFA ratio. A lower intake of nuts was observed for children with obesity.

It is important to note that although future nutritional intervention studies are necessary to better understand the impact of personalized diet on lipid metabolism in children, lipidomics can help monitor the Omega-6 fatty acid content involved in the inflammation pathways that can be accompanied by essential FA deficiencies in the diet, which can be connected to many diseases and tissue malfunctions.

As a matter of fact, monitoring the RBC membrane FA profile at the individual level can be an excellent candidate biomarker as it can offer the possibility to follow up the optimal intake, membrane incorporation, and biochemical transformations to personalize dietary intervention designed to recover FA deficiencies to prevent or control disease. Fatty acids in phospholipids represent the combination between nutritional and metabolic factors, with a strong contribution of the individual metabolism and condition of the patients.

3.2 The case of seed germination

Seed germination is a crucial event in the plant life cycle and, depending on seed quality, this step can severely constrain crop yield with a negative impact on the food chain. As soon as dry seeds are imbibed, complex events take place with precise temporal dynamics, leading to metabolism resumption. The seed coat is a barrier against oxygen diffusion; however the level of oxygen partial pressure (pO2) can differentially influence germination rates. Park and Hasestein [94] investigated the response of Brassica seeds under different pO_2 conditions, showing the high degree of seed adaptability in terms of gene expression and metabolite composition. Upon germination, the progressive depletion of oxygen generates conditions that almost achieve anaerobiosis and fermentation is triggered as the main source of cellular ATP, supporting the reduction of electron transferring compounds, e.g. NAD and NADP, and inevitably leading to ROS (reactive oxygen species) accumulation. At this stage, mitochondria, peroxisomes and the plasma membrane NADPH oxidases are the main sources of ROS, together with lipid catabolism and lipid β-oxidation in the

glyoxysomes. ROS levels must be strictly controlled, by means of antioxidant systems, and maintained within a defined range (the so-called “oxidative window”) to avoid any damage to the embryo that would impair germination. When germination starts, nitric oxide (NO) production is induced under oxygen limitation. NO turnover, by means of RNS (reactive nitrogen species) scavenging mechanisms, contributes to maintain a redox balance within the seed.

Upon rehydration, membrane reorganization anticipates the other molecular events and features the transition from the so-called hexagonal II phase to a lamellar phase that allows to restore the normal function, preventing leakage of cellular components. Membrane structure and domain organization strictly depends on lipid biochemical properties and composition. Indeed, the size of the polar head, compared to the hydrophobic tail, affects the lipid behavior in water. Indeed, lipids with a small polar head are characterized by a large negative curvature that promotes membrane organization in the form of inverted micelles (hexagonal II phase). These domains are mainly required to maintain membrane architecture and, within a certain group of lipids, features as increasing chain length and unsaturation number, facilitating the occurrence of hexagonal II phase. Lipids that own similar cross-section area at the level of the polar head and hydrophobic tail, resembling cylinders, from plan lamellar phases. Changes in environmental temperature can affect the proper membrane reorganization during imbibition, impairing germination rates. The chilling-imbibitional damage has been investigated by using lipidomics in maize seeds, revealing that the ability to germinate under cold stress is associated with phospholipid remodeling. On the other hand, global rise in temperature is a severe threat to crop productivity, causing a heat stress-mediated decline in germination rates. Membrane thermal stability combined with an effective antioxidant response, is a main component of cellular tolerance to heat stress.

Cellular lipids, particularly polyunsaturated fatty acids (PUFAs), are ROS-sensitive targets and the accumulation of their peroxidation products represent a major cause of decreased seed quality, in terms of germinability and longevity. Due to the cytotoxic effects of hydrogen peroxide (H_2O_2) on PUFAs, early seed imbibition is associated with lipid peroxidation that induce membrane damage and malondialdehyde (MDA) production. Lipid peroxidation causes of the so-called imbibition damage occurring when high rainfall induces rapid water up-take by dry seeds after sowing in the field or during imbibitional chilling. Imbibition damage is one of the major factors affecting seed quality in grain legumes, and it has been reported that predisposition to imbibition damage positively correlates with the presence of unpigmented testae. Mechanisms underlying the repair of damaged membranes in plants exposed to stress and even during seed imbibition are still poorly known;

although, showed that, in Arabidopsis, triacylglycerols can sequestrate toxic lipid intermediates, such as free fatty acids, providing protection against oxidative damage.

Within the complex lipid metabolism, the overall profile of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and PUFAs reflects the multiple membrane arrangements allowing optimized fluidity and permeability as well as suitable receptor- and channel-mediated functions. Under physiological conditions, the proper functions of the cell membrane are strongly dependent on the qualitative and quantitative balance of fatty acids.

Therefore, a thorough study of the fatty acid changes during the early step of seed germination should help understanding whether the seed metabolism is oriented towards the increase in unsaturated moieties that influence membrane properties. In this scenario, the impact of oxidative stress can be also evaluated by monitoring trans-fatty acids (TFAs) accumulation. TFAs, stereoisomers of the naturally occurring cis-fatty acids, are characterized by a linear configuration favoring intermolecular chain-chain interaction stronger than their natural cis counterparts. The occurrence of trans-fatty acid isomerization has been correlated to oxidative stress mechanisms mediated by sulfur-containing peptides and proteins, and the small diffusible thiyl radicals (RS•) resulting from the degradation of methionine, hydrogen sulfide (H_2S) and metal-sulfur clusters. Under reductive stress conditions, desulfurization at the protein level generates small sulfur-centered radicals that diffuse into the lipid bilayer causing rapid reactions with the PUFA residues and generation of TFAs can be regarded as putative biomarkers of oxidative stress. The isomerization catalyzed by thiyl radicals is a very efficient process, extensively investigated through mechanistic and kinetic studies, deriving from damaged sulfur-containing proteins diffused within the cellular environment. This event results in a further wave of changes by causing cis-to-trans isomerization of membrane lipids. However, at the moment, it is not yet well understood whether the process of cis-to-trans isomerization of membrane lipids represents exclusively a form of cellular damage, or it eventually acts as a signal leading to the activation of endogenous defense systems.

During seed imbibition, lipids are engaged in membrane reorganization while facing free radical-mediated oxidative injury. In frame of the study carried out with the group of A. Balestrazzi of University of Parma, we explored changes in lipid components at different timepoints of imbibition (0.5, 2, 4, 6, and 8 h) in the legume Medicago truncatula, by combining biochemical approaches with targeted lipidomics and untargeted metabolomics. ROS and RNS (reactive oxygen and nitrogen species) accumulation was observed throughout the tested timepoints whereas lipid peroxidation increased at 4 h of imbibition. The seed response to oxidative damage was evidenced by a significant

increase in tocopherols starting from 0.5 h of imbibition as well as by the reduction in total thiol content occurring at 2 h of imbibition. Since under physiological conditions, the proper functions of the cell membranes are strongly dependent on the qualitative and quantitative balance of fatty acid residues in phospholipids, the investigation was expanded to the fatty acid cohort of M. truncatula seeds. Total saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), polyunsaturated fatty acids (PUFAs), omega(ω)-3 and omega(ω)-6 fatty acids showed fluctuations during seed imbibition. The most remarkable finding was the profile of the ω-3 PUFA docosapentaenoic acid (DPA, 7 cis, 10 cis, 13 cis, 16 cis, and 19 cis-22:5) that showed a peak (up to 1.0% of the total fatty acid content) at 0.5 and 8 h of imbibition, concomitant with the peaks observed in tocopherol levels. It is possible that the observed changes in DPA alter the physical properties of membranes, as reported in animal cells, triggering signaling pathways relevant for the cell defense against oxidative injury. Furthermore, the content and balance between tocopherols and PUFAs is regarded as a determinant of storage stability. No enhancement in trans-fatty acids occurred throughout imbibition, suggesting for a proper antioxidant response carried by the seed. Fatty acids profiles were integrated with data from untargeted metabolomics showing changes in lipid sub-pathways, among which fatty acid amide, lyso-phospholipids and phospholipid metabolism.

4. Membrane technology in drug delivery: liposome formation

Lipid-based self-assembling vesicles were first described in 1961 and reported in 1964 by Dr. Alec D. Bangham [95] at the Babraham Institute in Cambridge, UK, in which he wrote: “It is probable that at equilibrium each and every lipid bilayer forms an unbroken membrane-there being no exposed hydrocarbon/water interface-from which it follows that every aqueous compartment would be discrete and isolated from its neighbor, including a complete separation of the outermost compartment of the whole structure from the continuous phase in which it is suspended”. It is thermodynamically possible for each lipid bilayer to form a discrete membrane, separating the vesicle from the continuous aqueous phase in which it is suspended. These “unbroken membranes” were called as “liquid crystal” or “smectic mesophase”, “Bangasomes” after the name of Dr. Bangham, and finally “liposomes”. The word “liposome” derives from two Greek words of “lipo-meaning fat” and “soma-meaning body”. In the earlier days, these artificial vesicles (or liposomes) were used for the study of cell

physiology such as ion (or drug) permeability, membrane fusion, membrane-bound enzyme properties or as a membrane model. More recently, more and more attention is brought to the uses of liposomes in medical fields as drug delivery systems and the first article of this kind was published in 1971 in FEBS Letters by Dr. George Gregoriadis and his co-workers, where amyloglucosidase and albumin were entrapped into liposome for the purpose of enzyme replacement therapy [96]. Since then, tremendous amounts of papers were published on the uses of liposomes as a drug delivery systems.

4.1 Testing liposome formulation for peptide delivery: the case of somatostatin

Somatostatin (also known as somatotropin release-inhibiting factor or growth hormone release-inhibiting factor, SST) is a cyclic peptide of 14 amino acids first isolated from ovine hypothalamic and known to inhibit the secretion of multiple hormones (e.g., growth hormone, insulin, glucagon, gastrin), gastric acid and pancreatic enzymes. In the central nervous system, this peptide acts as a neurotransmitter and affects locomotor activity and cognitive functions [97]. SST exerts its activity by binding to at least five different subtypes of specific receptors (SSTR 1-5) located on the target cells with a wide range of biological effects that can be exploited for the treatment of a variety of human diseases [98]. Signaling pathways activated by the SST-receptor interaction (such as, mitogen-activated protein kinase pathway, inhibition of adenylyl cyclase, activation of phosphotyrosine phosphatase, changes in plasma membrane calcium and potassium channel activity) are evoked for the antineoplastic and anti-proliferative activities [99]. The therapeutic potential of SST is strongly limited by its very short half-life of less than 1–2 min in plasma [100], as expected for several neuropeptide hormones that must be rapidly inactivated after their release and interaction with their receptors. In fact, in neuronal cell cultures the degradation of SST was measured, and membrane-bound proteases or proteases released into the incubation medium were found to be responsible for this inactivation [101].

Solutions to the short lifetime have been proposed based on two main approaches: (a) preparation of SST analogues attaching one or more groups to the peptide molecule, such as the N-methyl group, able to act as a shield for the in vivo hydrolysis [102]; (b) encapsulation of SST or its analogues in polymeric materials, such as poly(alkyl cyanoacrylate) nanocapsules [103], or in natural phospholipid vesicles, eventually coated with agents ensuring circulation in blood, such as polyethylene glycol (PEG), that protects liposomes from recognition and rapid removal from circulation by the phagocyte

system [104]. SST, its analogue octreotide or other synthetic analogues are also studied in liposomal formulations combined with antitumoral drugs like daunorubicin or with radiopharmaceuticals, due to their ability to target SSTR-rich tumoral cells [105]. The biocompatibility and biodegradability of liposomes have an overwhelming importance thus motivating research to deepen the use of natural phospholipids as drug delivery components. It is worth noting that, as far as natural lipid formulations are concerned, the choice of the fatty acid quality is important for liposome behavior, since it influences fluidity and permeability properties connected with drug release [106].

Multilamellar liposomes made of natural phosphatidylcholine were used for the incorporation of a mixture of somatostatin and sorbitol dissolved in citrate buffer at pH = 5. Lyophilization and reconstitution of the suspension were carried out, showing the flexibility of this preparation. Full characterization of this suspension was obtained as particle size, encapsulation efficiency and retarded release properties in aqueous medium and human plasma.

The release experiment was designed to analyze not only the directly released SST (in the aqueous phase of the nanoemulsion (NE)—direct analysis) but also the amount of non-released SST, still in the lipid droplets (indirect analysis), Figure 9 A. Since SST can have stability problems, the determination of the peptide remained in the lipid phase was meant to collect important additional information for the characterization of the release profile of the NE.

In Figure 9 B it is clearly shown that the peptide is diminishing along the time when considering as starting quantity the encapsulated SST (0.5 mg/mL).

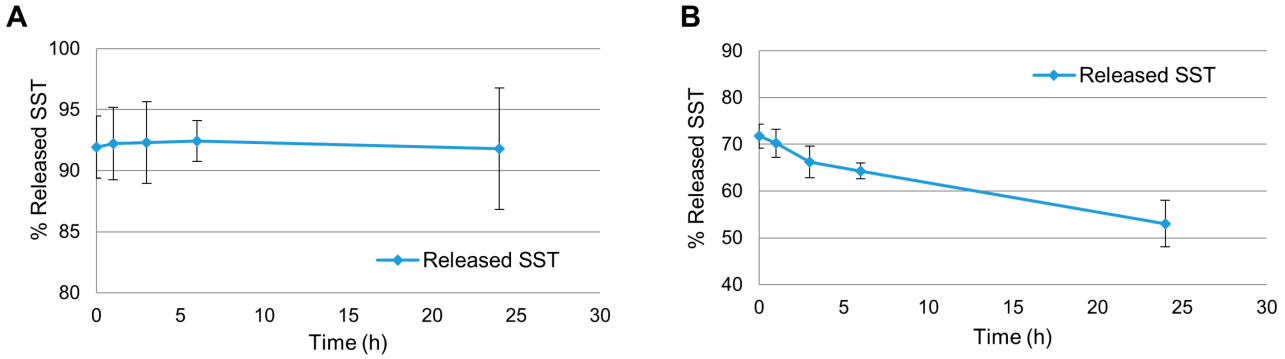


FIG 9: Released somatostatin (SST) from the nanoemulsions (NEs) in 0.1 M buffer citrate pH 5.0. (A) % released SST considering the sum of the SST fraction found in aqueous phase (direct analysis) and the SST fraction in the lipid droplets (indirect analysis) at each time point. (B) % released SST in the aqueous phase considering as 100% the initial amount of SST used in the preparation procedure (0.5 mg/mL).

The experiments of the NE release using human plasma were run following two protocols. The first procedure consisted of reconstituting the freeze-dried NE directly in plasma to start the release

experiment (Figure 10 A). In the second one, the NE was first reconstituted in water, then underwent centrifugation to remove the non-encapsulated fraction of SST, and the resulting pellet was suspended in human plasma to start the experiment (Figure 10 B).

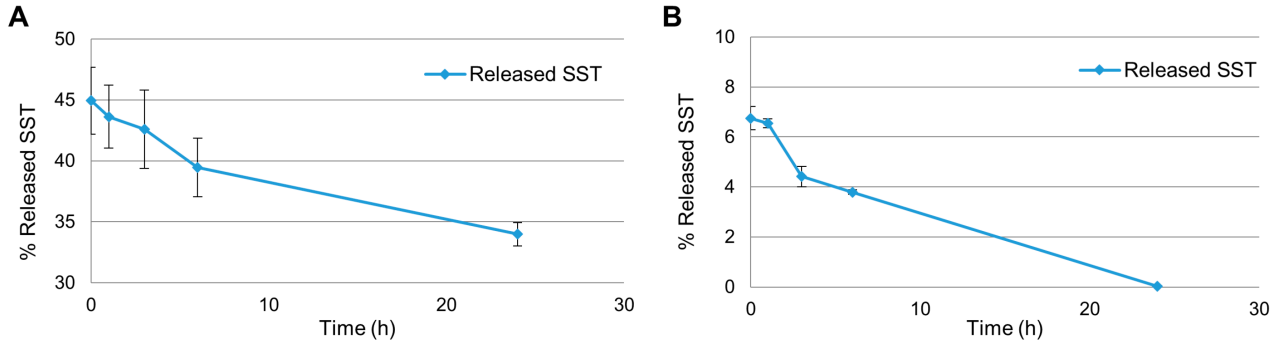


FIG 10: (**A**) Measurement of SST concentration in plasma after reconstituting the NEs directly in plasma (protocol 1). (**B**) Measurement of SST concentration in plasma after removing the non-encapsulated SST fraction (protocol 2). Percentages are calculated from the LC/MS analyses related to the starting concentration of 0.5 mg/mL

In (Figure 10 A), the amount of SST measured in the aqueous phase of the NE was 45% of the starting concentration (0.5 mg/mL) at time 0 and decreased to 34% after 24 h. When the NE release profile was studied following protocol 2, roughly 7% of the SST was found in plasma at time 0, and after 24 h only traces of SST were found (0.03%) (Figure 10 B). These results imply that there was a decrease in concentration due to degradation of the peptide. Nevertheless, SST is known to have very short half-life in plasma and the NE proved to be efficient in improving the peptide half-life, maintaining its measurable concentration at least for the first 7–8 h.

These results are promising for application as a drug delivery system by extension to appropriate experiments in biological context, using cells or murine models.

Nutraceutical development and marketing application

1. Nutraceuticals

Nutraceutical is a term derived from the combination of the words “nutrition” and “pharmaceutics.” The term is applied to products that are isolated from herbal products, dietary supplements (nutrients), specific diets, and processed foods such as cereals, soups, and beverages that other than nutrition are also used as medicine. [107]

In Italy, the term “nutraceutical” products are regulated as drugs, food ingredients and dietary supplements. The term is not defined the same in different countries but is usually defined as a product isolated from foods that is generally sold in medicinal forms not usually associated with food. A nutraceutical product may be defined as a substance, which has physiological benefit or provides protection against chronic diseases. [107] Nutraceuticals may be used to improve health, delay the aging process, prevent chronic diseases, increase life expectancy, or support the structure or function of the body.

Nutraceuticals, in contrast to pharmaceuticals, are substances, which usually have not patent protection. Both pharmaceutical and nutraceutical compounds might be used to cure or prevent diseases, but only pharmaceutical compounds have governmental sanction [108].

A dietary supplement is considered as a product that bears or contains one or more of the following dietary ingredients: a mineral, a vitamin, an amino acid, a medical herb or other botanical, a dietary substance for use by man to supplement the diet by increasing the total daily intake, or a concentrate, metabolite, constituent, extract, or combinations of these ingredients. Nutraceuticals are of these nutritional supplements which are used for health purposes other than nutrition. [109]

Some popular nutraceuticals include ginseng, *Echinacea*, green tea, glucosamine, omega-3, lutein, folic acid, and cod liver oil. Majority of the nutraceuticals possess multiple therapeutic properties.

Nowadays, nutraceuticals have received considerable interest due to potential nutritional, safety and therapeutic effects. A market research recently proposed that the European nutraceuticals market is expanding and would reach USD $158 billion by 2025. [110]

1.1 DHA

The ω-3 fatty acid DHA belongs to the family of ω-3 PUFAs, essential components of cell membranes, which play many important biological functions. In vivo, DHA is produced through a nine-step biosynthetic pathway, requiring elongation and desaturation reactions, which starts from α-linolenic acid, its essential precursor introduced by the diet.

The efficiency of the biosynthetic pathway has different degrees of efficiency in humans, therefore the semi-essential nature of DHA is emerging. DHA has gained increased interest over the years, as multiple roles in various molecular pathways and signaling networks have been described in the last decades. Noteworthy, all biological activities are dependent on the naturally occurring polyunsaturated cis geometry [111].

As a sign of its recognized importance in biological functions, the main international food safety and health agencies established that 100-200 mg are an adequate daily intake from dietary sources, such as algae or fish, especially for children and pregnant women [112]. This led to an increased marketing of DHA-rich formulas, both as functional foods and as nutraceuticals.

1.2 Importance of DHA

DHA (4c,7c,10c,13c,16c,19c, docosahexaenoic acid) is a long chain polyunsaturated fatty acid (LC-PUFA) belonging to the ω-3 series [113]. Concerning its chemical structure, DHA is a carboxylic acid characterized by 22 carbon atom chain having 6 double bonds in cis position (Figure 11). The last double bond is located three carbons from the methyl end, thus it is a so-called ω-3 fatty acid.

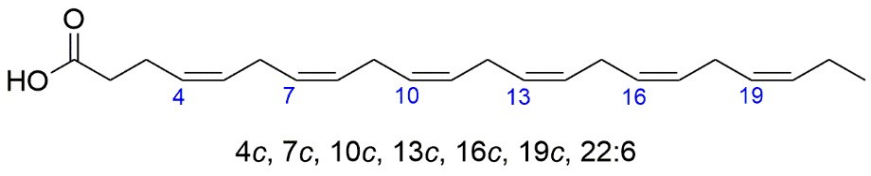


FIG 11: Chemical structure of DHA.

Among the PUFAs present in the human body, DHA has the longest acyl chain and the higher number of double bonds. DHA can be endogenouly synthesized through enzymatic pathways, or come from dietary exogenous sources.

There are some controversies about the mechanism of DHA biosynthesis in mammals. From a synthetic point of view, DHA can be synthesized de-novo from its precursor, α-linolenic acid, that is an essential fatty acid, through a series of elongation and desaturation steps that alternatively occur in the endoplasmatic reticulum.

Initially, it was hypothesized that DHA could be directly formed from its immediate precursor 22:5ω3 by the action of a Δ4 desaturase that introduced a double bond in position Δ4. In lower eukaryotes, like Thraustochytrium sp, the existence of a Δ4-desaturase has been demonstrated [20], but the presence of this enzyme in mammals has never been ascertained [114].

An alternate route, that involves peroxisomes, known as the "Sprecher pathway", has been generally accepted [115]. This pathway requires an elongation step to convert 22:5 ω3 to 24:5 ω3, followed by a second Δ6-desaturation step to 24:6 ω3; the product would finally be β-oxidated in the peroxisome to produce DHA (Figure 1.12).

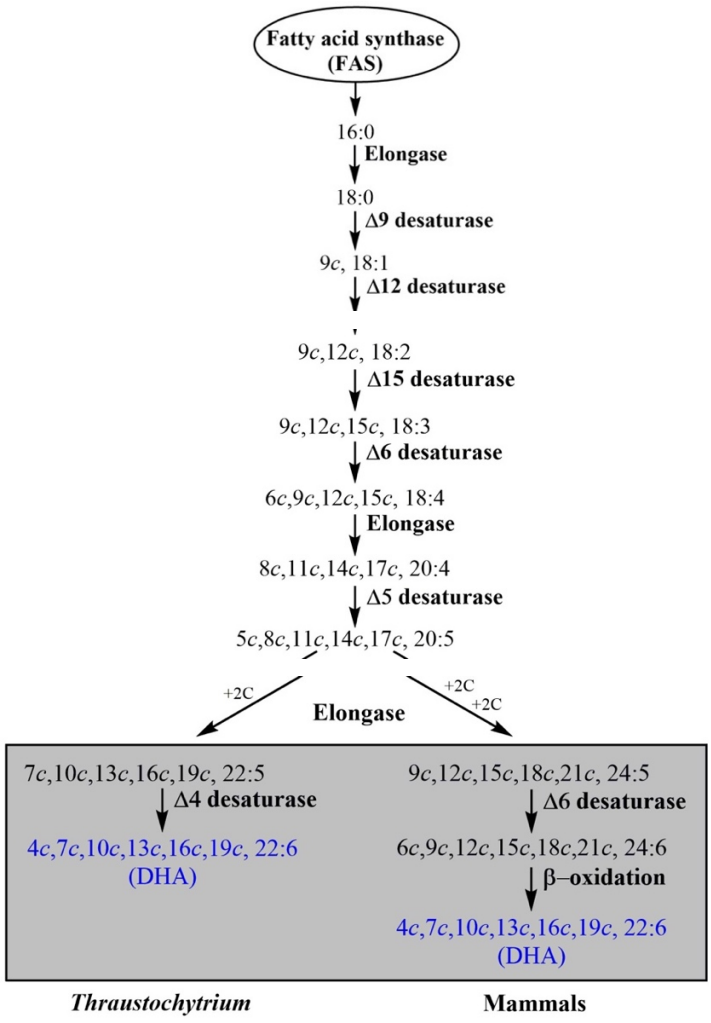


FIG 12: Enzymatic pathways for the synthesis of DHA in Thraustochytrium and mammals.

De-novo synthesis of DHA is influenced by several factors that modify the catalytic activity of some of the enzymes involved in the process; moreover, some of these enzymes compete for different PUFAs, such as the the ω-6 chain ones.

The desaturation step, carried out by Δ6 desaturase is the first and rate-limiting step of the PUFA synthesis [51]. This enzyme, in a similar way as Δ5-desaturase, is inhibited by cholesterol, SFAs and TFAs [116]. Additionally, the activity of both desaturases was found to be reduced by alcohol, glucocorticoids, and adrenaline [117] and in hyperlipidemia and hypertension conditions [118].

Finally, competition between α-linolenic acid and 24:5 ω3 for the enzyme could explain the reduction in DHA levels observed following α-linolenic acid intake beyond certain values [119].

These costraints made it necessary to increase DHA assumption through the diet. In this regard, DHA can be found in discrete quantities in fish, particularly in salmon, mackerel, sardines, herring, tuna and anchovies [120]. Fish meat is enriched with DHA because a preferential fish food is microalgae, such as Crypthecodinium Cohnii, Schizochytrium and Ulkenia, which constitute the main source of DHA in nature [121]. Apart from these foods, DHA's dietary sources are particularly limited. Only small amounts of DHA are found in meat and in eggs, especially if the animal has been fed algae-based fodder [122].

DHA plays a crucial role in the human body. It is found in high levels in the neuronal system and in the retina because it is essential for nervous neurotransmission and visual development [57] [123]. For this reason, appropriate amounts of this fatty acid must be introduced during pregnancy and breast feeding to guarantee the proper childhood development. Considerably high levels of DHA are needed in the pregnancy period that immediately precedes birth, that is when cerebral growth occurs and DHA is required for rapidly expanding neuronal cell biosynthesis [124]. Studies report that children which are nor breastfed nor supplemented with DHA enriched formulas show mental retardation in comparison with children which received appropriate supplies of DHA. Nutraceutical supplementations in ω-3 fatty acids are a new trend in the prevention approach, to ensure the proper development of brain tissue, visual function and cognitive ability of fetuses and newborns. Moreover, in children affected by attention deficit and hyperactivity disorder, DHA supplementation was reported to reduce aggressivity and improve learning and social relationship ability [125] [126].

Although the molecular mechanism is still unknown, DHA is hypothesized to modulate neurotransmission by improving signal transduction processes and ensuring optimum communication between nerve cells: in fact, a study on patients admitted for severe depression and other behavioral

disorders (bipolarism and schizophrenia), shows that a balanced ω-3 regime reduces the frequency and intensity of depressive events and stabilized the mood [127].

As mentioned above, recent studies from our group on the cellular membrane of autistic children, DHA deficiency was observed and associated with the loss of function of Na^+ / K^+ ATPase of the erythrocyte membrane [76]. Several studies highlighted the possible role of DHA in inhibiting the progressive neurodegeneration, such as Alzheimer's disease, that may occur in the elders [124]. Concerning visual diseases, DHA can prevent senile degeneration of the macula in adults [128].

DHA is also present in considerable quantities in sperm cells, where it participates in the regulation of spermatogenesis and membrane fluidity to guarantee spermatozoa migration from seminiferous tubules to epididymis [129].

Additionally, DHA can be found in the cardiac tissue, where it is considered to reduce triglycerides in the blood. DHA enriched diets were reported to be beneficial for cardiopathic and hypertensive patients, decreasing cardiovascular risk factors, such as atherosclerosis and hypertension that are responsible for heart attacks and stroke [130] [131]. Clinical benefits of dietary supplementation with DHA are reported for people suffering from rheumatoid arthritis [132]and ulcerative colitis [133]. Regulation of cell growth by DHA can have a significant impact on the development of certain types of tumors, such as colorectal, prostate and breast cancer [134] [135] [136].

Therefore, studies concerning DHA, its chemical transformations and nutraceutical use are of great interest not only for scientific research, but also for industrial and therapeutic applications.

1.3 Food Sources

There are many dietary sources of omega-3 FAs including fish, krill, algae, and land plants [137]. The type and amount of DHA varies between sources. Fish is the most common source of omega-3 FAs and the amount of DHA varies between fish species, time of year, the fish’s diet, and geography. Cold water, pelagic fish usually have the highest levels of DHA. Overall, in marine fish the most important factor is their total fat content, with high fat fish having the highest amount of omega-3s per serving. Sardine, mackerel, herring, and halibut have some of the highest omega-3 PUFA levels but are uncommon in many diets [137]. In the United States, salmon, anchovies, herring, sardines, Pacific oysters, trout, and Atlantic and Pacific mackerel are the most commonly consumed low mercury seafood varieties [138].

The frequent consumption of fish does raise some safety and environmental concerns. Fish is susceptible to bioaccumulation of toxins and pollutants, one of the most common being mercury [133]. An advantage of using Fish Olis (rather than consuming whole fish) is that oil refining removes the majority of these toxins. Another concern is overfishing of the supply that could strain the sustainability of the market [139].

Alternative marine sources are available as a source of omega-3 FA, without facing some of the challenges associated with using fish. Krill oil can achieve higher levels of EPA and DHA than FO but the product has a higher cost so it is usually used in supplements [137] . In addition, there are marine plant sources of omega-3 FAs that can be used commercially in food products. Algae are primary producers of omega-3 FAs, which can be cultivated to produce a continuous supply of omega-3 FAs. While algae produce high amounts of DHA. Until recently, relatively high production and purification costs limited the large scale manufacturing of algae oils, however, considerable advances have been made in recent years that have led to their increased commercial use [140].

The Dietary Guidelines and American Dietetic Association encourage nutrient consumption from food rather than supplements however, people may choose to consume supplements or fortified foods for many reasons including cost, their dislike of seafood, allergies, a vegan diet, convenience, and the inability to meet recommended DHA levels from their normal diet [139]. Consumers seeking alternative sources of omega-3 should be aware if the products contain ALA, EPA or DHA in order to receive maximum health benefits.

Land plant sources of omega-3 FAs include canola, soy, flaxseed, and walnuts mainly in the form of ALA [139]. An increased consumption of omega-3 fatty acids from these sources may have a limited effect in decreasing cardiovascular disease or a stroke because of the inefficient conversion of ALA to EPA and DHA [140].

Supplements may contain EPA and DHA in different forms than the common triglyceride form typically found in Fish Oils [137]. Ethyl esters of omega-3 FAs are commonly used in dietary supplements and pharmaceuticals because of the ability to distill the ethyl esters and produce highly concentrated oils [137]. The ethyl esters of DHA have a different absorption route in the human body than triglyceride forms, but plasma lipid levels appear to be equivalent, however the triglyceride form can be better utilized in the body [137].

1.4 Fish oil

In the remainder of this section, we will primarily focus on fish oils as it is considered to be the most common, least expensive, and best source of DHA in the human diet [141]. However, other sources

of omega-3 fatty acids are becoming more economically viable, such as genetically engineered oil seeds [142].

Industrial production of Omega-3 fish oils

In the industrial process, the unpleasant fishy smell of fish oils is eliminated by deodorization, using high temperature and low-pressure procedures, however such conditions have been found to affect the natural cis structure of the PUFAs, converting them to the geometrical unnatural trans isomers (Paragraph 1.1). The dire consequences of trans PUFA uptake for health have been discovered two decades ago and nowadays the presence of trans fats in foods is banned in the USA and is matter of careful evaluation in Europe [143].

In the frame of ISOF group research on free radical modification of biomolecules, they studied TFAs obtained by sulfur-centered radical-induced isomerization process occurring with an addition-elimination mechanism, where no double bond shift was taking place, so that only trans geometrical isomers were produced.

The strategy consisted in the comparison of mono-trans isomers obtained by free radical-catalysed isomerization with mono-trans isomers isolated and characterized as mono-epoxide precursors (Figure 13). The separation and assignment of the mono-epoxide structures successfully provided the first unambiguous determination of the trans alkene position, thus integrating data already present in the literature [144] [145] [146].

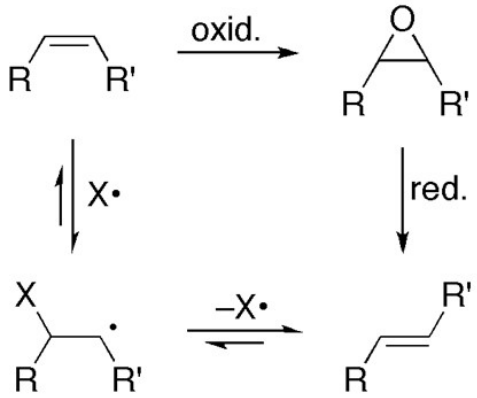


FIG 13: Dual synthetic approach from cis-alkene to obtain the geometrical trans isomer.

They applied the same analytical approach for the synthesis and characterization of the mono-trans DHA isomers [147].

The isomeric mono-trans DHA mixture was characterized by GC analysis and this GC library was used to evaluate the presence of mono trans-DHA in many Italian, Spanish and Japanese commercially available fish oil-containing capsules.

FAME*	1	2	3	4	5	6
14:0	5.75±0.04	0.56±0.01	6.11±0.08	6.01±0.05	0.81±0.03	7.58±0.12
16:0	12.86±0.01	1.45±0.05	22.54±0.15	13.14±0.08	23.48±0.08	19.21±0.14
16:1 trans-Δ^9	0.34±0.02	0.07±0.01	0.64±0.06	0.46±0.00	nd	0.52±0.02
16:1 cis-Δ6+Δ7	0.16±0.01	0.03±0.00	0.26±0.05	0.15±0.01	nd	0.31±0.01
16:1 cis-Δ9	6.75±0.05	2.31±0.07	9.05±0.03	5.02±0.04	0.44±0.00	9.46±0.06
18:0	3.00±0.05	1.44±0.06	3.17±0.05	3.86±0.01	1.48±0.12	4.07±0.13
18:1 trans-Δ9	0.11±0.03	0.04±0.00	0.11±0.00	0.09±0.00	nd	0.13±0.00
18:1 trans-Δ11	0.06±0.02	0.01±0.00	0.62±0.01	0.02±0.00	nd	0.08±0.00
18:1 cis-Δ9	8.41±0.04	10.09±0.10	26.70±0.10	6.39±0.13	0.02±0.00	10.43±0.05
18:1 cis-Δ11	2.73±0.09	3.59±0.07	5.48±0.03	2.41±0.05	0.56±0.04	3.61±0.11
18:2 trans-Δ12	nd	0.13±0.01	0.07±0.00	nd	0.03±0.00	0.01±0.00
18:2 trans-Δ9	nd	0.05±0.02	0.03±0.00	0.02±0.00	0.08±0.00	0.03±0.00
18:2 ω6	1.36±0.05	2.18±0.09	1.83±0.04	1.08±0.01	0.14±0.00	1.45±0.01
18:3 ω6	0.32±0.08	0.29±0.03	0.03±0.00	0.43±0.00	nd	0.30±0.02
18:3 ω3	0.58±0.06	0.98±0.08	0.69±0.03	0.49±0.01	nd	0.93±0.11
20:0	0.22±0.02	0.13±0.00	0.14±0.00	0.37±0.00	0.31±0.01	0.50±0.05
20:1	0.98±0.08	1.06±0.04	7.75±0.05	1.44±0.12	nd	1.52±0.00
20:3 ω6	0.25±0.09	0.41±0.04	0.07±0.00	0.27±0.00	0.21±0.01	0.23±0.04
20:4 trans-Δ14	0.04±0.00	0.01±0.00	nd	0.03±0.00	nd	0.03±0.03
20:4 ω6	1.95±0.07	2.35±0.07	0.19±0.01	1.82±0.10	0.13±0.00	1.31±0.11
20:4 other mono-trans	0.04±0.00	0.06±0.00	nd	0.05±0.00	nd	0.04±0.02
20:3 ω3	0.12±0.00	0.17±0.00	0.12±0.01	0.10±0.01	nd	0.10±0.02
20:5 ω3 trans-Δ17	0.13±0.00	0.09±0.01	0.01±0.00	0.12±0.03	nd	0.03±0.00
20:4 ω3	1.08±0.04	2.05±0.06	0.64±0.07	1.14±0.01	0.91±0.01	0.95±0.01
20:5 ω3	28.05±0.28	44.34±0.12	3.47±0.05	27.40±0.15	0.66±0.01	20.53±0.26
20:5 ω3 other mono-trans	0.62±0.02	0.07±0.00	0.02±0.00	0.56±0.00	nd	0.06±0.00
22:0	0.09±0.02	0.08±0.00	0.04±0.00	0.13±0.01	0.20±0.01	0.14±0.00
22:1	0.12±0.01	0.08±0.00	2.06±0.05	0.28±0.06	nd	0.28±0.03
22:5 ω6	0.70±0.03	0.69±0.00	nd	0.55±0.00	11.91±0.13	nd
22:6 ω3 trans-Δ19	**0.12±0.01**	**0.06±0.00**	**0.53±0.03**	**0.13±0.08**	**0.12±0.04**	**0.03±0.00**
22:5 ω3	3.67±0.07	4.31±0.02	0.77±0.05	3.91±0.18	0.15±0.02	2.44±0.14
22:6 ω3	18.51±0.10	20.64±0.37	3.69±0.04	20.98±0.07	57.35±0.15	12.98±0.15
22:6 ω3 trans-Δ4	**0.08±0.00**	**0.01±0.00**	**0.43±0.03**	**0.07±0.00**	**0.17±0.00**	**0.06±0.05**
22:6 ω3 trans-Δ13	**0.19±0.02**	**0.02±0.00**	**0.44±0.02**	**0.14±0.03**	**0.33±0.01**	**0.03±0.02**
24:1	0.34±0.03	0.10±0.00	1.30±0.00	0.66±0.01	nd	0.57±0.10
22:6 ω3 trans-Δ7+Δ10	**0.26±0.04**	**0.02±0.00**	**1.00±0.01**	**0.29±0.02**	**0.50±0.02**	**0.05±0.04**
Total SFA	21.92±0.05	3.66±0.10	32.00±0.17	23.52±0.14	26.28±0.21	31.49±0.13
Total MUFA	19.49±0.20	17.27±0.23	52.60±0.14	16.34±0.06	1.03±0.04	26.18±0.03
Total PUFA	55.89±0.25	77.72±0.37	11.51±0.22	57.61±0.05	59.53±0.19	41.22±0.11
PUFA ω6	4.58±0.20	5.93±0.16	2.12±0.05	4.15±0.09	12.39±0.14	3.29±0.10
PUFA ω3	52.01±0.31	72.48±0.51	9.38±0.18	54.01±0.06	59.06±0.18	37.93±0.15
Total TFA	2.00±0.07	0.66±0.04	3.89±0.10	1.98±0.15	1.25±0.02	1.12±0.13
TFA ω3	1.40±0.10	0.27±0.01	2.43±0.09	1.30±0.14	1.13±0.03	0.27±0.08

TAB 1: Fatty acid content (%rel) determined in omega-3 containing supplements commercially available in Italy. Analyses performed in triplicates (n=3)

FAME*	7	8	9	10	11	12	13	14	15	16	17	18	19
14:0	0.11±0.00	0.67±0.01	9.39±0.09	8.85±0.04	8.73±0.04	7.28±0.05	0.03±0.00	0.01±0.00	0.01±0.00	10.24±0.19	0.04±0.00	0.18±0.01	0.29±0.00
16:0	0.55±0.01	13.78±0.06	18.15±0.19	19.43±0.13	17.72±0.07	17.68±0.13	0.10±0.00	0.06±0.00	0.06±0.00	42.32±0.69	0.18±0.01	1.02±0.01	1.02±0.18
16:1 trans-Δ^9	0.01±0.00	0.38±0.03	0.62±0.04	0.59±0.03	0.45±0.03	0.52±0.02	nd	nd	nd	0.63±0.03	0.01±0.00	0.03±0.00	nd
16:1 cis-Δ6+Δ7	0.01±0.00	0.19±0.02	0.22±0.03	0.28±0.02	0.21±0.01	0.24±0.02	nd	nd	nd	0.07±0.01	0.02±0.00	0.01±0.00	nd
16:1 cis-Δ9	0.37±0.00	7.21±0.03	11.14±0.07	10.92±0.04	11.59±0.05	8.88±0.01	0.09±0.00	0.03±0.00	0.01±0.00	3.73±0.32	1.06±0.03	0.53±0.01	0.77±0.03
18:0	6.16±0.03	3.40±0.09	3.41±0.05	3.87±0.01	3.25±0.01	3.91±0.01	0.06±0.00	0.04±0.00	0.03±0.00	1.97±0.17	0.11±0.01	6.51±0.03	2.25±0.14
18:1 trans-Δ9	0.13±0.01	0.25±0.09	0.15±0.03	0.17±0.03	0.15±0.03	0.14±0.04	nd	0.01±0.00	nd	0.08±0.02	0.01±0.00	0.15±0.01	nd
18:1 trans-Δ11	0.07±0.02	0.22±0.05	0.11±0.04	0.10±0.04	0.03±0.01	0.13±0.02	nd	nd	nd	0.01±0.00	0.01±0.00	0.09±0.00	nd
18:1 cis-Δ9	10.25±0.01	17.78±0.06	9.14±0.02	9.09±0.00	8.58±0.01	9.08±0.06	0.24±0.00	0.12±0.00	0.04±0.00	9.04±0.03	7.13±0.06	10.63±0.15	nd
18:1 cis-Δ11	4.19±0.06	6.27±0.04	3.55±0.09	3.72±0.03	3.44±0.04	3.15±0.06	0.03±0.00	0.16±0.00	0.01±0.00	5.53±0.02	2.76±0.05	4.61±0.04	nd
18:2 trans-Δ12	nd	0.21±0.04	0.08±0.01	0.16±0.02	0.15±0.01	0.17±0.03	nd	nd	nd	0.01±0.00	0.27±0.02	0.12±0.02	nd
18:2 trans-Δ9	0.03±0.01	0.60±0.03	0.04±0.00	0.04±0.01	0.04±0.01	0.06±0.01	nd	0.01±0.00	nd	0.03±0.00	0.16±0.01	0.26±0.04	5.61±0.21
18:2 ω6	1.54±0.00	3.40±0.05	2.27±0.04	1.79±0.03	1.53±0.02	4.94±0.02	0.05±0.01	0.13±0.00	0.05±0.01	2.07±0.08	1.47±0.02	2.35±0.01	nd
18:3 ω6	0.25±0.01	0.56±0.06	0.26±0.03	0.30±0.01	0.35±0.04	2.14±0.07	0.03±0.00	nd	nd	0.14±0.02	0.28±0.03	0.28±0.00	nd
18:3 ω3	0.95±0.01	1.53±0.02	0.72±0.04	0.81±0.03	0.81±0.02	0.81±0.02	0.02±0.00	nd	nd	0.85±0.14	0.87±0.03	1.17±0.01	nd
20:0	0.64±0.01	0.24±0.03	0.18±0.02	0.36±0.02	0.31±0.01	0.48±0.03	0.04±0.00	nd	nd	0.08±0.01	0.06±0.00	0.75±0.01	0.30±0.01
20:1	2.79±0.07	0.91±0.03	0.85±0.02	1.14±0.05	0.64±0.04	1.81±0.04	0.08±0.00	0.02±0.00	nd	0.56±0.05	0.47±0.04	2.75±0.03	0.35±0.11
20:3 ω6	0.46±0.02	0.21±0.01	0.20±0.03	0.18±0.03	0.23±0.03	0.19±0.04	0.03±0.00	0.01±0.00	nd	0.07±0.02	0.40±0.03	0.48±0.02	0.23±0.00
20:4 trans-Δ14	0.06±0.01	0.05±0.04	0.03±0.00	0.02±0.00	0.02±0.00	0.01±0.00	nd	0.03±0.00	nd	0.01±0.00	0.04±0.01	0.09±0.02	nd
20:4 ω6	2.27±0.03	1.49±0.03	1.17±0.05	1.19±0.03	1.73±0.01	1.22±0.01	0.12±0.00	0.72±0.00	0.02±0.01	0.28±0.01	2.71±0.04	2.32±0.06	0.48±0.01
20:4 other mono-trans	0.04±0.00	0.05±0.04	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00	nd	0.01±0.00	nd	0.02±0.00	0.05±0.02	0.03±0.00	nd
20:3 ω3	0.17±0.03	0.13±0.01	0.07±0.01	0.09±0.02	0.07±0.02	0.11±0.01	0.02±0.00	nd	nd	0.09±0.00	0.11±0.03	0.19±0.00	nd
20:5 ω3 trans-Δ17	0.06±0.01	0.11±0.01	0.03±0.00	0.05±0.01	0.06±0.01	0.05±0.01	0.03±0.00	0.01±0.00	0.03±0.01	0.03±0.00	0.08±0.00	0.09±0.02	nd
20:4 ω3	1.73±0.02	0.86±0.03	0.74±0.04	0.90±0.02	0.80±0.02	0.92±0.02	0.09±0.00	0.06±0.00	0.09±0.01	0.33±0.01	1.95±0.01	1.65±0.03	nd
20:5 ω3	37.56±0.22	21.87±0.19	20.24±0.21	20.00±0.14	21.41±0.11	19.60±0.19	2.29±0.00	3.24±0.01	5.59±0.01	13.48±0.34	48.32±0.17	34.95±0.15	2.35±0.03
20:5 ω3 other mono-trans	0.07±0.01	0.14±0.02	0.10±0.02	0.06±0.00	0.09±0.01	0.09±0.00	0.04±0.00	0.07±0.01	0.12±0.01	0.14±0.19	0.09±0.00	0.11±0.00	nd
22:0	0.29±0.01	0.08±0.01	0.84±0.07	0.12±0.02	0.11±0.01	0.15±0.01	0.06±0.00	0.02±0.01	nd	0.07±0.01	0.02±0.00	0.28±0.02	0.55±0.08
22:1	0.37±0.02	0.16±0.02	0.14±0.02	0.17±0.02	0.09±0.01	0.44±0.02	0.15±0.00	nd	nd	0.45±0.01	0.03±0.00	0.34±0.03	nd
22:5 ω6	nd	nd	nd	nd	nd	nd	1.97±0.00	6.24±0.01	0.52±0.03	nd	0.64±0.03	0.78±0.04	3.92±0.80
22:6 ω3 trans-Δ19	**0.06±0.01**	**0.09±0.01**	**0.04±0.01**	**0.04±0.01**	**0.05±0.00**	**0.05±0.02**	**0.27±0.03**	**0.34±0.03**	**0.31±0.02**	**0.02±0.00**	**0.06±0.01**	**0.07±0.00**	**0.66±0.24**
22:5 ω3	5.20±0.06	2.10±0.03	2.11±0.06	2.13±0.02	2.18±0.01	1.98±0.00	9.94±0.32	1.17±0.02	13.32±0.04	0.38±0.02	3.96±0.02	4.47±0.02	2.09±0.33
22:6 ω3	22.60±0.14	14.60±0.15	13.50±0.10	12.90±0.10	14.71±0.05	13.11±0.06	83.68±0.17	86.19±0.11	78.78±0.11	7.16±0.31	26.54±0.09	21.85±0.07	76.75±0.41
22:6 ω3 trans-Δ4	**0.02±0.01**	**0.08±0.00**	**0.03±0.01**	**0.03±0.00**	**0.03±0.01**	**0.05±0.01**	**0.09±0.03**	**0.16±0.02**	**0.18±0.03**	**0.02±0.00**	**0.02±0.00**	**0.02±0.01**	**0.30±0.01**
22:6 ω3 trans-Δ13	**0.02±0.00**	**0.09±0.01**	**0.03±0.00**	**0.02±0.00**	**0.03±0.02**	**0.02±0.01**	**0.10±0.04**	**0.46±0.03**	**0.35±0.02**	**0.02±0.00**	**0.05±0.00**	**0.07±0.04**	**0.74±0.02**
24:1	0.97±0.03	0.24±0.01	0.40±0.00	0.47±0.04	0.31±0.02	0.54±0.03	0.14±0.03	nd	nd	0.04±0.00	0.01±0.00	0.75±0.07	0.34±0.14
22:6 ω3 trans-Δ7+Δ10	**0.03±0.01**	**0.07±0.00**	**0.02±0.00**	**0.02±0.00**	**0.07±0.02**	**0.04±0.01**	**0.20±0.03**	**0.68±0.01**	**0.49±0.03**	**0.01±0.00**	**0.02±0.00**	**0.02±0.00**	**1.00±0.14**
Total SFA	7.74±0.03	18.17±0.12	31.96±0.23	32.62±0.14	30.12±0.09	29.50±0.17	0.30±0.00	0.13±0.01	0.10±0.01	54.68±0.71	0.40±0.00	8.73±0.07	4.40±0.22
Total MUFA	18.95±0.16	32.75±0.04	25.45±0.15	25.78±0.10	24.86±0.05	24.12±0.15	0.73±0.03	0.33±0.01	0.06±0.01	19.43±0.29	11.46±0.09	19.62±0.09	1.47±0.19
Total PUFA	72.71±0.20	46.75±0.31	41.29±0.30	40.30±0.10	43.83±0.06	45.02±0.11	96.27±0.14	91.52±0.09	97.85±0.11	24.85±0.66	86.61±0.14	69.72±0.17	81.90±0.61
PUFA ω6	4.51±0.07	5.66±0.07	3.90±0.07	3.46±0.09	3.84±0.08	8.49±0.14	2.19±0.01	7.10±0.01	0.59±0.04	2.57±0.12	5.50±0.12	6.21±0.11	4.64±0.81
PUFA ω3	68.20±0.26	41.09±0.36	37.39±0.29	36.84±0.19	39.99±0.13	36.53±0.23	96.05±0.14	90.66±0.10	97.78±0.11	22.28±0.78	81.76±0.22	64.29±0.19	81.18±0.61
Total TFA	0.60±0.05	2.33±0.26	1.31±0.09	1.30±0.13	1.19±0.08	1.36±0.14	0.72±0.10	1.78±0.08	1.47±0.09	1.04±0.22	0.88±0.07	1.14±0.08	8.32±0.35
TFA ω3	0.26±0.03	0.57±0.04	0.24±0.01	0.22±0.02	0.34±0.06	0.30±0.05	0.72±0.10	1.72±0.08	1.47±0.09	0.24±0.19	0.32±0.01	0.38±0.03	2.71±0.39

TAB 2: Fatty acid content (%rel) determined in omega-3 containing supplements commercially available in Spain. Analyses performed in triplicates (n=3) of the same sample

1.5 Dietary recommendations for LC-PUFA

Many organizations at the national and international levels have published recommendations for Omega-3 fatty acids. These recommendations vary in the specificity of omega-3 FA forms taken, such as fish, ALA, EPA, and DHA, and if subsets of the general population require different recommendations. In the United States, the 2010 Dietary Guidelines for Americans suggests consuming 250 mg of EPA and DHA per day. It is recommended that pregnant women consume from 227 to 340 g of low mercury seafood per week [138].

The *National Academies* (USA) has made its omega-3 FA recommendations using adequate intake values. An adequate intake value is used if a recommended daily allowance cannot be established and is determined based on the intake of healthy people (4). For males and females 14 years old and above, the adequate intake value of ALA, EPA and DHA are 1.6 and 1.1 g/day, respectively with most of the recommendation coming from ALA [148]. Pregnant and lactating women have an adequate intake value of 1.4 and 1.3 g omega-3s/day, respectively.

The American Dietetic Association and Dieticians of Canada recommend 2 servings of fatty fish per week; 500 mg of EPA and DHA per day [149]. The American Diabetes Association suggest at least 2 servings of fish per week for adequate omega-3 FA consumption [150]. Commercially fried fish filets are excluded from this recommendation. The American Heart Association recommends 2 servings of fatty fish per week in order to obtain beneficial amounts of EPA and DHA [151].

The European Food Safety Agency proposes the dietary intake of 250-500 mg of EPA and DHA/day for adults [152]. They also acknowledge that supplementing up to 1 g of DHA per day is safe. The Scientific Advisory Committee on Nutrition of Great Britain recommends at least 2 servings of fish (140 g) per week with at least one of the servings from oily fish. In France, the French National Nutrition and Health Program (PNNS) recommends eating fish two times a week . The French Food Safety Agency (AFFSA) recommends that individuals over the age of 10, including pregnant and lactating women, should consume 500 mg of EPA and DHA/day and a minimum of 250 mg of DHA/day [153].

The World Health Organization recommends 2 servings of fish per week in order for the consumer to intake about 200 to 500 mg of EPA and DHA per day [154]. The Australian and New Zealand National Health and Medical Research Council recommends 430 and 610 mg/day of DHA/EPA/DPA (docosapentaenoic acid) for women and men between the ages of 19 and 69 years [155]. For pregnant and lactating women from 19-50 years old, 115 and 145 mg/day of DHA/EPA/DPA is recommended.

Western diets in general do not provide satisfactory omega-3 FA intakes. American's current consumption of EPA and DHA is lower than the recommended values. On average, Americans are currently consuming 100 g of seafood per week and much of it is low in omega-3 FAs. The National Health and Nutrition Examination Survey (NHANES) determined the mean intake of EPA and DHA through food sources by people over 19 years is 23 and 63 mg/day, respectively. For individuals over the age of 19 consuming EPA and DHA through both food and supplement sources, they are consuming 41 and 72 mg/day, respectively. As of the 2008/2009 and 2010/2011 surveys, the actual consumption of oily fish by the population of Great Britain was not meeting the recommendation [156]. On average, only 54 g of oily fish were consumed per week across the age range of 19-64 years. Adults over 65 consumed an average of 90 g of oily fish per week. In contrast, Japanese diets easily provide sufficient omega-3 fatty acids. The Japanese population achieves the recommended intake values of DHA and EPA through their diet high in seafood, and their use of dietary fats high in ALA [157]. Japanese adults consume about 80 g of fish and shellfish per day, resulting in around 1-2 g of omega-3 FA per day.

Although many of the dietary recommendations for omega-3 encourage consumption of fish, this is not always convenient: some people do not like fish; some people cannot afford fish; fresh fish spoils

rapidly; fish may contain undesirable contaminants (such as heavy metals); overfishing may reduce the supply of fish available; the growing global population puts a higher demand on the available fish. Consequently, there is great interest in the development of alternative means of incorporating omega-3 fatty acids into the human diet.

2. Nutrilipidomic approach

"-Omics" technologies have an important role in the comprehension of metabolism and signaling pathways at a molecular level, aiming at envisaging early stages of malfunctioning and disease onsets, as well as contributing to the advancement of molecular diagnostics and biomarkers for health care and disease prevention [158].

The "-Omics" approach monitors molecular levels and activities of genes, proteins, carbohydrates, lipids and all their metabolites, offering a systematic view of the most relevant biological pathways and responses. This includes effects of nutritional elements. On this basis, it is timely to develop medical approaches where the "molecular" status of the patient is evaluated during the anamnesis, coupled with clinical observation and set-up of a personalized therapeutic strategy, including dietary intervention. Genomics in the nutritional area created the field of nutrigenomics dealing with the effects of nutritional elements on gene expression and transcription factors, however the number of studies is still considered limited to allow for a full application to medical care [159].

On the other hand, lipidomic profiling is an important tool to explore the impact of nutrition and metabolism [160], being fatty acid- containing molecules one of the most important lipidomic targets. This subject connects technological and analytical advancements of the last decade to decades of biochemical and nutritional research on fatty acids (FAs), highlighting their important roles for the membrane phospholipid structures and functions, the regulatory and signaling networks, the activation of specific receptors, the influence on expression of genes and protein responses.

Moreover, a full scenario of the type and quantity of FAs coming from the interplay of biosynthesis and diet is also available. The essentiality of omega-6 and omega-3 polyunsaturated fatty acids (PUFA) for eukaryotic cells (i.e., linoleic acid and α-linolenic acid) and the role of specific lipid enzymes, namely desaturases and elongases, have been thoroughly studied. The essentiality of FAs has been recognized in medicine (e.g., dermatology, ophthalmology, and cardiology) and fascinating involvements of FA pathways have been discovered for various pathologies (e.g., cancer, obesity, diabetes, and neurodegeneration) [161]. Also, the market developed, since the inputs of these biological and medical research generated an exponential growth of formulations containing FAs and related cofactors, which are used as nutraceuticals or dietary supplements. In Europe, following the

Regulation 1924/2006, the European Food Safety Agency (EFSA) has started to revise scientific basis for health claims of nutritional supplements, and the effects of FAs have been obviously included in the survey. A few health claims have been accepted so far, creating concerns in the criteria used to assess the effects and in the future directions of supplement design and production.

Dr. Carla Ferreri and Dr. Chryssostomos Chatgilialoglu, founders of Lipinutragen Srl, coined the term "nutrilipidomics" for an innovative tool for personalized health care [162]. In this case, a specific lipid pool belonging to the cell membrane compartment of a specific tissue is the target, i.e., the FA composition of membrane phospholipids as a comprehensive indicator of metabolic and nutritional effects. The drawing in Figure 1 shows the context of nutrilipidomics having the FA content of cell membranes at the crossroad of various contributions.

As mentioned above the membrane FA asset, i.e., saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), present in the phospholipids is characteristic of each tissue. A natural adaptation response is active, and the appropriate changes of the FA microenvironment ensure the best functioning of membrane proteins, receptors, pumps and signals in tissues, according to environmental and metabolic needs. Therefore, FA status is dynamic including the remodeling that follows the propagation of stimuli, where FAs are continuously released from membranes to act as mediators and specific ligands. Membrane composition is also related to a homeostatic control that keeps constant the working environment of protein, channels and receptors by a meticulous FA balance, involving hydrolase, esterase and transferase enzymes.

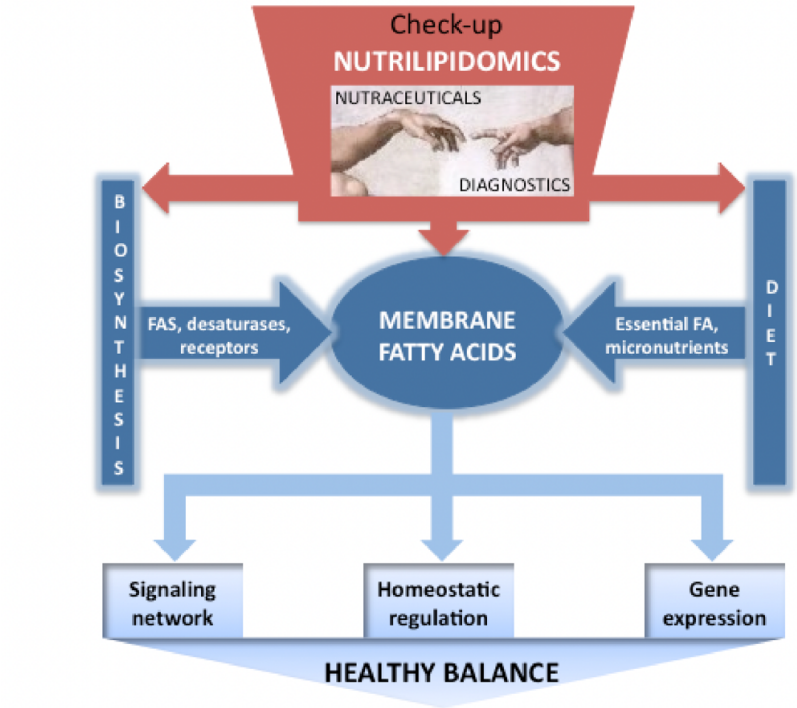


FIG 14: The Nutrilipidomic Approch for a re-equilibrium strategy [163]

Figure 14 can evidence failures of the FA availability and, exploiting the efficiency of the natural lipid remodeling and the cell turnover, suggests a targeted re-equilibrium strategy for restoring this functional pool. Membrane lipidomics is the basic tool of the nutrilipidomic approach to target personal needs and use the full benefits of a nutrition-based strategy. The choice of the cell for nutrilipidomics is based on the presence of all representative fatty acid families and the reasonable cell lifetime for evaluating the turnover and the acquired balance, together with the influence of correction factors.

In erythrocyte membranes a meaningful cohort of FAs can provide the basic data set [162]. This panel also includes FA ratios and enzymatic indexes, calculated by the relative ratios among fatty acid families and substrates: the ratio SFA/MUFA can indicate biosynthetic and dietary contribution, which create the balance between these two FA families in the membrane compartment, regulating structural organization, biophysical properties and functioning. From changes at molecular level most of the harmful consequences of SFA for health can indeed arise [164]. In this respect the role of unsaturations, namely the natural double bond content, is extremely important for favorable membrane biophysical properties of fluidity and permeability, as well as for biochemical functions and signaling cascades. It is worth underlining that the unsaturated content is correlated to the functioning of enzymes, namely Δ5-, Δ6- and Δ9-desaturases. These enzymes work regio- and stereo-specifically, meaning that the position and geometry of the double bonds are fixed and have defined biological meanings. Obviously, the dietary supply of MUFA and (PUFA) residues regulates these enzymatic activities, and a critical interpretation of the individual status can be carried out combining with complete anamnesis and dietary habits of the subject. Evaluation of desaturase genes can be effected by genomics [165] and in parallel by enzymatic indexes in the lipidomics of erythrocyte membranes. Nowadays these parameters are used as risk factors for several diseases [166] [167].

The effects of FAs as essential elements for health generated a tremendous increase of commercial products containing plant and animal oils as mentioned above. The use of these supplements can occur without medical prescription and a strong advertisement campaign on the nutraceutical effects for skin, muscle, liver, brain, heart, joints health, together with slowing aging, rendered them the products of choice by millions of consumers, considering them as natural, therefore safe, substances. Nutrilipidomics can have an important role in rendering this choice personalized and adequate to personal needs, being evident the strict relationship between the status verified by membrane lipidomics and the type and dosage to be used. Indeed, it must be considered that hydrophobic substances like FAs accumulate especially following high dosages for long periods. This can certainly influence the FA pools with modifications also of the membrane content. Such changes should not

be random but strategically used for improving the individual condition. Another related aspect is the reactivity of unsaturated FAs with free radicals, which can occur by two main processes: peroxidation and isomerization. Oxidized and trans-FA are a large variety of molecules with many biological effects, but their excess has been recognized to be harmful for health [168]. Research have already evidenced that lipid peroxidation can be indeed connected with the use of PUFA supplementation.

With the nutrilipidomics approach FAs will be fully employed as nutraceuticals. Indeed these substance satisfy the three basic requisites for nutraceuticals: (i) they are needed for humans, their intake is also from external sources, that is a must for essential fatty acids; (ii) possible scarce intake or consumption by known modes can lead to impairment of their levels in the body and, consequently, of most tissues and metabolic pathways; (iii) their supplementation can ensure health of tissues and functions. However, to be considered nutritional elements with pharmaceutical (health) effects, i.e., nutraceuticals, they must have a precise site of action and a specific type and dosage for efficacy. Indeed, protocols reporting the FA supplementation have never been run by considering personalized treatments or dosages, neither upon verification of the oxidative and metabolic conditions, as well as their starting FA profile. This methodological issue can probably explain the variability and contradictions of the effects described in the literature, which created also doubts in the effectiveness of the "lipid therapy". In the approach of nutrilipidomics, the use of erythrocyte membranes to evaluate the subject's status and the type of personalized intervention create the evidence-based choice of supplementation and the dosage tailored on the subject's conditions. [168]

Another element of the nutrilipidomics approach is to bring innovation for the nutraceutical design. Databases of membrane lipidomic analyses can be organized in order to individuate profiles of subjects having different health conditions. The multivariate analysis for lipidomics profiles can be also implemented with parameters derived from other "-omics" and molecular diagnostics. In this way, nutrilipidomics can motivate an innovative productive chain for nutraceutical lines customized on the basis of the "-omics" profiles, offering its scientific support to the identification of effective formulations and dosage in nutraceuticals.

This approach can be very useful for societal needs and consumers: the need of a market of nutraceuticals proposing products based on real needs, thus becoming closer to consumers, and the timely establishment of health care operators able to couple the patient's clinical conditions with the corresponding molecular profile as a rationale for personalized intervention including nutritional elements. Overall, nutrilipidomics is expected to boost personalized health with an ideal balance between scientific evidences and market sustainability.

3. Emulsions

Emulsions are dispersed biphasic systems or are a mixture of two immiscible liquids, in which one of the phases, called dispersed phase, is represented by droplets separated from each other by more or less wide regions occupied by molecules of the other phase, called dispersing phase. The dispersing phase is also called the continuous phase, it determines the macroscopic physical properties of the entire system. Emulsions are thermodynamically unstable systems.

They are obtained through the mechanical agitation of the two liquids that will form them; in fact, the mechanical work translates into the work of extending the separation surface, which is directly proportional to the surface tension at the interface between the dispersed phase and the continuous phase.

Emulsions are very complex systems (from finely to coarsely dispersed). If there is a dispersion of oil in water (O / W), the character of the emulsion is given by the water, that is, by the continuous phase, such as milk. In this case, the dispersed phase is of a lipidic nature and the continuous one is made up, instead, of water.

Water-in-oil (W / O) emulsions behave in the opposite way to that described, such as margarine.

The droplet concentration is expressed as a mass or volumetric fraction and can be calculated by centesimal, density or composition analysis. By already knowing the density of the different phases, we pass from mass to volume and the opposite.

The emulsions are therefore characterized as a function of the concentration of the droplets and their size.

The size of the droplets, in fact, govern the external appearance and based on them we speak of macro and microemulsions.

Microemulsions are characterized by droplet diameters of less than 0.5 μm, optically transparent and thermodynamically stable. Macroemulsions, on the other hand, have larger droplets, up to a maximum of 100 μm [169].

Emulsions with small droplets are more viscous than those with large droplets [170]. The emulsions are further differentiated into monodisperse emulsions, if the droplets of the dispersed phase are all the same size, and into polydisperse emulsions, otherwise. The dimensions of the particles constituting the dispersed phase are of considerable importance, because they influence numerous characteristics of food emulsions, such as texture, flavor, stability, shelf life, production costs and many other aspects.

The structure of an emulsion can be represented by a dispersed phase, in the form of more or less small droplets, by a continuous phase and by an interface region, as shown in Figure 15.

The particles present in the emulsion are associated with the continuous or dispersed phase or are arranged along the interface region. This depends on their affinity for one of the three regions and, therefore, on their concentration and polarity.

The non-polar molecules tend to arrange themselves in the lipid phase, the polar ones in the aqueous phase, while the amphiphilic molecules arrange themselves in the interface region by turning the polar portion of the molecule towards the aqueous phase and the apolar towards the lipid phase.

At the interface of the two immiscible liquids, free energy is developed resulting from the non-balancing of the cohesion forces.

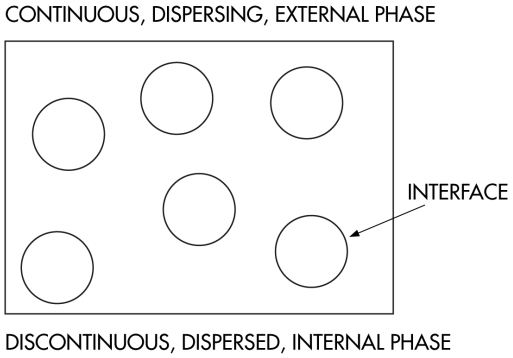


FIG 15: Graphic representation of the continuous, discontinuous phase and the interface region of the emulsion.

This energy is called interfacial energy or tension and tends to form the smallest specific interface surface [171].

As previously mentioned, the emulsion is obtained by means of mechanical agitation, as it is necessary to supply energy by subjecting the liquids to violent agitation, so as to impart shear stress (but also compression, impact and cavitation) to the phase portions internal to deform and divide them progressively.

The forces known as Laplace pressure oppose the deformation and breaking of the particles of the dispersed phase:

$$\Delta p = \frac{2\gamma}{R}$$

where Δp represents the Laplace pressure (Pa), γ the surface tension (N / m2) and R (m) represents the diameter of the droplets.

To break up the drops of the dispersed phase, an energy must therefore be applied that develops a Δp greater than the Laplace pressure [171]. The smaller the drops, the more Δp increases and the more the applied force must increase to obtain a further subdivision of the drops that make up the dispersed phase.

It is possible to obtain an emulsion through homogenization or through emulsification, a less severe operation than the previous one. Both operations lead to the reduction of the particle size (to 0.5-3 μm) of the dispersed phase, through the application of intense shear forces, to increase the intimacy and stability of the two substances; if you want to obtain an extreme degree of dispersion in an emulsion, homogenizers are used.
These unitary operations have the purpose of changing the overall structure and sensory properties of foods, while they do not cause any effect on nutritional values or shelf-life.
The equipment used is classified into distinct groups of high-speed mixers:
1. pressure homogenizers;
2. colloidal mills;
3. ultrasonic homogenizers.
Obtaining a good emulsion depends on several factors during preparation, such as the temperature which must be the same in the two phases, the stirring speed and the stirring time.
The emulsions are inherently unstable because the mixture tends to decrease its energy by decreasing the interface area.

In particular, those prepared by simple emulsification of two immiscible liquid phases, constitute a very unstable system as the droplets of the dispersed phase, by virtue of their high surface energy, tend to reunite (coalesce) and decrease in number until they form a single phase with minimal contact surface area.
To prevent this phenomenon and, therefore, to stabilize the emulsions it is necessary to introduce a third component.
This can be represented by additives, specifically emulsifiers, which through the formation of a film cover the surface of the drops and raise an energy barrier that prevents the drops of the dispersed phase from aggregating, or by thickeners, additives used to increase the viscosity of the continuous phase of the emulsions and to improve the stability of the emulsions, delaying the movements of the droplets.
The emulsifiers can be divided into ionic and non-ionic surfactants, however they are able to form a monomolecular film that surrounds the droplets of the dispersed phase and have both an effect on interfacial tension and a barrier effect that prevents coalescence. Today the most used are non-ionic surfactants.
A good emulsifying agent should:
1) have affinity for both phases, that is, it must be constituted by amphiphilic molecules, which possess polar, hydrophilic, and non-polar, lipophilic regions;

2) possess surfactant properties so as to reduce interfacial tension;
3) adsorb easily and quickly to the surface of the dispersed droplets, forming a film capable of preventing coalescence;
4) imparting a charge to the droplets so as to favor electrostatic repulsion;
5) act effectively at low concentrations.
These additives are characterized by a numerical value called HBL (hydro lipophilic balance) which derives from the ratio between the hydrophilic portion and the lipophilic portion of the molecule.
The HBL value can vary from 1 to 40, depending on this value the use of the different emulsifiers is established.
The emulsifiers currently used in food production are purified natural products or synthetic chemicals, which have structures very similar to natural products.
Just like any other food additive, emulsifiers are subject to strict legislation in the EU, which regulates the evaluation of their safety, authorizations, methods of use and labeling (Directive 1333/2008 of the European Parliament on food additives other than dyes and sweeteners).
The regulation requires that all added emulsifiers, like all food additives, be declared on the packaging with their name or with the identification number preceded by the letter E.
The factors that contribute to stabilizing the emulsions are:

1) emulsifying agent: as already said, it is generally a surfactant, which by lowering the interfacial tension decreases the free energy of the system; alternatively, non-surfactant substances can also be used, such as gum arabic, gelatin, hydrophilic colloids or finely divided powders (for example talc). These substances are distributed at the O / W interface forming a protective film, more rigid than that formed by surfactants, which forms a barrier to coalescence. On the other hand, substances with a strong affinity for water (such as sugars) can remove water from the interfaces by breaking the emulsion.
2) barrier at the interface: the surface defined by the encounter between the continuous phase and the droplets that make up the discontinuous phase, is electrostatically charged regardless of the ionic or non-ionic nature of the surfactant. In fact, the charge is due either to the polar nature of one of the two phases, or to friction (which generates an electrostatic charge), or to the adsorption of ions present inside the solution (even the surfactants are arranged at the interphase for adsorption). It is therefore evident that ionizable substances (such as salts) can interfere with surface charges by decreasing repulsive forces and facilitating coalescence.
3) viscosity of the medium, sedimentation or skimming speed, particle size: these factors are correlated to each other by Stokes' law:

$$v = \frac{2R^2\ (d_e - d_i)g}{9\eta}$$

where v represents the sedimentation speed, R the radius of the dispersed particles, d the density of the internal phase, d the density of the external phase, g the acceleration of gravity, η the viscosity of the continuous phase. From this law it can be deduced that the more stable the system will be the more similar the density of the two phases is and the greater the viscosity of the continuous phase; furthermore, an attempt will be made to reduce the size of the particles as much as possible, optimizing their dimensions (with subsequent experimental tests) according to the surfactant used that controls their stability. The stability of the emulsions is not thermodynamic but kinetic, this means that the separation of the phases takes place in the emulsion after a certain time, more or less long. The main modifications that lead to the decay of the emulsion are mainly given by the phenomena of creaming and sedimentation, flocculation, coalescence and phase inversion. Creaming or surfacing occurs when the droplets, having a low density compared to the surrounding liquid, rise to the surface.

Sedimentation, on the other hand, represents the arrangement on the bottom of a certain number of droplets with higher density.

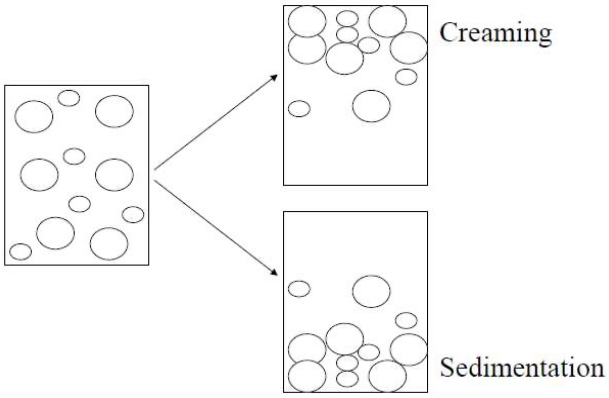


FIG 16: Effect of gravitational force on fluids of different density outcrop and sedimentation.

We talk about flocculation when several original particles form a cluster (the floccule), thanks to interparticle bonds that affect the respective active sites, while maintaining their respective identity (flakes of denatured proteins following heat treatment of the corresponding colloidal solution).

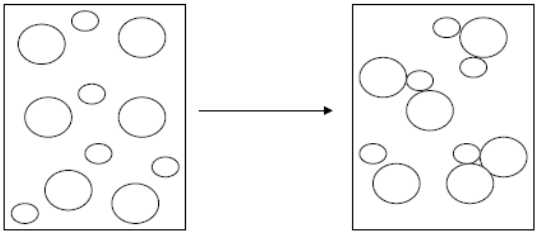


FIG 17: Effect of the interaction energy between the two particles as a function of distance: flocculation.

We talk about coalescence when more particles merge to form one of greater mass and it is no longer possible to recognize the original particles (a liquid drop that forms from smaller droplets).

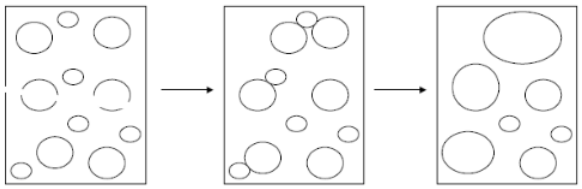


FIG 18: Effect of the interaction energy between the two particles as a function of distance: coalescence.

By phase inversion we mean the conversion of an oil-in-water emulsion into another of the water-in-oil type and vice versa. It hardly occurs during storage, but it can occur in the emulsification phase and requires high temperatures.

Ultimately it is possible to state that the stability of an emulsion depends on various and numerous factors, including the degree of dispersion of the internal phase (variability in diameter), the nature of the interface, viscosity of the dispersing phase, phase / volume ratio, specific weight of the two phases and others.

3.1 Microemulsions

If a surfactant that possesses balanced hydrophilic and lipophilic properties is used in the right concentration, a different oil and water system will be produced. The system is still an emulsion, but exhibits some characteristics that are different from the milky emulsions discussed previously. These new systems are called “microemulsions”. The interfacial tension between phases, amount of energy required for formation, droplet sizes, and visual appearance are only a few of the differences seen when comparing emulsions to microemulsions.

Microemulsions are in many respects small-scale emulsions. They are fragile systems in the sense that certain surfactants in specific concentrations are needed for microemulsion formation [172]. In simplest form, they are a mixture of oil, water, and a surfactant. The surfactant, in this case, generates

an ultra-low free energy per unit of interfacial area between the two phases (10^{-3} mN/m), which results from a precise balance between the hydrophilic and lipophilic nature of the surfactant and large oil-to-water interfacial areas. These ultra-low free energies allow thermodynamically stable equilibrium phases to exist, which require only gentle mixing to form [173]. This increased surface area would ultimately influence the transport properties of a drug [174]. The free energy of the system is minimized by the compensation of surface energy by dispersion entropy. The flexible interfacial film results in droplet sizes that fall in a range of 10-100 nm in diameter for microemulsion systems [173]. Although these systems are formed spontaneously, the driving forces are small and may possibly take time to reach equilibrium [174]. This is a dynamic process. There is diffusion of molecules within the microstructures and there are fluctuations in the curvature of the surfactant film. These droplets diffuse through the continuous phase while kinetics of the collision, merging, and separation of droplets occur [173]. With droplet sizes in the nanometer range, microemulsions are optically transparent and are considered to be solutions [175]. They are homogeneous on a macroscopic scale, but are heterogeneous on a molecular scale [176]. Microemulsions usually exhibit low viscosities and Newtonian flow characteristics. Their flow will remain constant when subjected to a variety of shear rates. Bicontinuous formulations may show some non-Newtonian flow and plasticity. Microemulsion viscosity is close to that of water, even at high droplet concentrations. The microstructure is constantly changing, making these very dynamic systems with reversible droplet coalescence [176]. To study the different properties of microemulsions, a variety of techniques are usually employed. Light scattering, x-ray diffraction, ultracentrifugation, electrical conductivity, and viscosity measurements have been widely used. These are only a few of the many techniques used to characterize microemulsions.

Types of Microemulsions

Microemulsions are thermodynamically stable but are only found under carefully defined conditions [177]. One way to characterize these systems is by whether the domains are in droplets or continuous [178]. Characterizing the systems in this way results in three types of microemulsions: oil-in-water (o/w), water-in-oil (w/o), and bicontinuous. Generally, one would assume that whichever phase was a larger volume would be the continuous phase, but this is not always the case.

Oil-in-water microemulsions are droplets of oil surrounded by a surfactant (and possibly co-surfactant) film that forms the internal phase distributed in water, which is the continuous phase. This type of microemulsion generally has a larger interaction volume than the w/o microemulsions [179]. The monolayer of surfactant forms the interfacial film that is oriented in a “positive” curve, where the polar head-groups face the continuous water phase and the lipophilic tails face into the oil droplets. The o/w systems are interesting because they enable a hydrophobic drug to be more soluble in an

aqueous based system, by solubilizing it in the internal oil droplets. Most drugs tend to favor small/medium molecular volume oils as opposed to hydrocarbon oils due to the polarity of the poorly water-soluble drugs. An o/w drug delivery tends to be straightforward when compared to w/o microemulsions. This is the result of the droplet structure of o/w microemulsions being retained on dilution with the biological aqueous phase [179].

Water-in-oil microemulsions are made up of droplets of water surrounded by an oil continuous phase. These are generally known as "reverse-micelles", where the polar headgroups of the surfactant are facing into the droplets of water, with the fatty acid tails facing into the oil phase. This type of droplet is usually seen when the volume fraction of water is low, although the type of surfactant also impacts this as well [179] A w/o microemulsion used orally or parenterally may be destabilized by the aqueous biological system. The biological system increases the phase volume of the internal phase, eventually leading to a "percolation phenomenon" where phase separation or phase inversion occurs [179] . Oral peptide delivery in w/o microemulsions is still used, however. The hydrophilic peptides can be easily incorporated into the water internal phase and are more protected from enzymatic proteolysis by the continuous oil phase than other oral dosage forms. A w/o microemulsion is best employed, though, in situations where dilution by the aqueous phase is unlikely, such as intramuscular injection or transdermal delivery [179].

When the amount of water and oil present are similar, a bicontinuous microemulsion system may result. In this case, both water and oil exist as a continuous phase. Irregular channels of oil and water are intertwined, resulting in what looks like a "sponge-phase" [180]. Transitions from o/w to w/o microemulsions may pass through this bicontinuous state. Bicontinuous microemulsions, as mentioned before, may show non- Newtonian flow and plasticity. These properties make them especially useful for topical delivery of drugs or for intravenous administration, where upon dilution with aqueous biological fluids, forms an o/w microemulsion [181].

Surfactant Use in Microemulsions

Surfactants are molecules that typically contain a polar head group and an apolar tail. They are surface-active and microstructure-forming molecules with a strong chemical dipole. They can be ionic (cationic or anionic), nonionic, or zwitterionic. Surfactant molecules self-associate due to various inter- and intra-molecular forces as well as entropy considerations. All of these serve to optimize the free-energy overall. For example, when surfactant is mixed with oil and water, they accumulate at the oil/water interface, because it is thermodynamically favorable [179] .The surfactant molecules can arrange themselves in a variety of shapes. They can form spherical micelles, rod-shaped micelles, a hexagonal phase (consisting of rod-shaped micelles), lamellar (sheet) phases, reverse micelles, or hexagonal reverse micelles [179]. At low concentrations of dispersed (internal)

phase, spherical, isolated droplets are present in the microemulsions. At higher dispersed phase concentrations, the final structures depend on the interaction between droplets. If they are repulsive, no droplet overlap will be produced due to colliding droplets. If attractive interactions are present, multiple droplets may collide and form other structures.

The hydrophilic-lipophilic balance (HLB) of the surfactant can be taken into account to try to rationalize the surfactant's behavior. It is generally accepted that a surfactant with HLB from 3-6 will favor the formation of water-in-oil (w/o) microemulsions, whereas surfactants with HLB from 8-18 are preferred for oil-in-water (o/w) microemulsions [179]. It must be noted, though, that microemulsions are only obtained under certain carefully defined conditions, and the HLB of the surfactant can only be used as a starting point in the selection of components that will form a microemulsion.

Another method used to relate the type of surfactant to the structures it forms is through the critical packing parameter (CPP). This, like HLB, is an empirical approach since there are many other factors that impact the final structures found in microemulsions. The CPP is a measure of the surfactant's preferred geometry, and therefore can be used to predict the type of structure that possibly will be formed. The CPP can be calculated by dividing the partial molar volume of the hydrophobic part of the surfactant by the product of the optimal headgroup area and length of the surfactant tail [179]. Surfactants that are "cone- shaped" where the tailgroup or headgroup is much larger than the other will tend to accumulate at curved interfaces resulting in micelles. Surfactants that are more "block-shaped" where tailgroup and headgroup are similar in size and the CPP values are close to one tend to form worm-like micelles or lamellar structures. Values of CPP greater than one indicate that the headgoups are much larger, resulting in w/o microemulsion systems. The opposite is true for CPP values less than one. They generally produce o/w microemulsion systems. Values for CPP around one indicate the possible formation of lamellar phases [179].

Regardless of the surfactant chosen for the microemulsion formulation, it must be able to lower the interfacial tension to an extremely small value. This aids the dispersion process, providing a flexible film that readily surrounds droplets of the internal phase while still having appropriate lipophilic character to provide a curvature at the interfacial region.

Nonionic Surfactants

Most nonionic surfactants are structurally similar to ionic surfactants, except for the fact that with ionic surfactants, the headgroup is uncharged. Because there are no electrostatic charges from the headgroups, the interactions between these nonionic headgroups are dominated by steric and osmotic forces [182]. Cosurfactants are generally not needed to form microemulsions with nonionics. This is due to the fact that pure specimens of nonionic's usually are made up of mixtures of slightly varying

chain length. Ethoxylated alcohols are the most common nonionic surfactants. These alcohols contain a wide-ranging degree of ethoxylation, where ethylene oxide is added to fatty acids to make them more water-soluble. They are considered “amphiphiles”, with an oil- loving hydrocarbon tail group and a water loving ethoxylated alcohol group. Nonionic surfactants show good biological acceptance [183]. They are able to form microemulsions that are insensitive to pH and electrolyte concentration [179] . Examples of nonionic surfactants include polyoxyethylene surfactants, such as Brij 35, or sugar esters, such as sorbitan monooleate (Span 80). Polyoxyethylene sorbitan monooleate (Tween 80) and polyoxyethylene sorbitan monolaurate (Tween 20) appear safe and acceptable for oral and parenteral use [179]. Polysorbates are partial fatty acid esters of sorbitol and its anhydrides copolymerized with approximately 20, 5 or 4 moles of ethylene oxide for each mole of sorbitol and its anhydrides. These vary in size due to a mixture of molecules and are considered hydrophilic nonionic surfactants. Sorbitans are partial esters of sorbitol and its mono and dianhydrides with fatty acids. These are considered lipophilic nonionic surfactants. Nonionic surfactants that contain sugar hydrophilic groups, such as alkylpolyglucoside surfactants, and sucrose ester surfactants are very hydrophilic and form temperature-insensitive microemulsions with the addition of alcohol. Alkanol amides and polyamines are the primary nitrogen-based nonionic surfactant types .

Ionic Surfactants

The use of ionic surfactants can be fairly limited in general pharmaceutical dosage forms. A large majority of ionic surfactants do not form balanced microemulsions without the addition of another component. These additives are required because the head group of the ionic surfactants are generally substantially more hydrophilic than poly(ethylene oxide) moieties. The salts or co-surfactants shift the overall HLB into the optimal range for microemulsion formulation . The addition of co-surfactants will be discussed in a later section.

Ionic surfactants can be cationic, anionic, or zwitterionic. Cationic surfactants generally fall into the class of quaternary ammonium alkyl salts. Alkylammonium halides and tetra- alkylammonium halides are the most numerous in this class. Alkyl ammonium halides are excellent hydrogen bond donors and interact strongly with water. The most well known examples from the cationic surfactant class are hexadecyltrimethyl-ammonium bromide (CTAB) and didodcecylammonium bromide (DDAB). Although less numerous, phosphorous can be quaternarized with alkyl groups to create alkyl phosphonium cationic surfactants as well [184].

Alkali alkanoates, also known as soaps, are the most common anionic surfactants. The anionic charge in these surfactants comes from the ionized carboxyl group. This type is the most well understood surfactant when it comes to their structure and function [184] . Dioctyl sodium sulfosuccinate (DOSS) is the most widely studied anionic surfactant. It has twin tails and is a particularly good stabilizer of

w/o microemulsions [179] . Other important classes of anionic surfactants include alkyl sulfates, alkyl ether sulfates, alkyl sulfonates, aryl sulfonates, methylester sulfonates, α-olefinsulfonates, and sulfonates of alkylsuccinates. The three most important anionic groups in all of these surfactants being the carboxylate, sulfate, and sulfonate groups [184].

Zwitterionic surfactants, which contain both negatively and positively charged groups, form microemulsions upon the addition of co-surfactants. Phospholipids, such as lecithin, are common zwitterionic surfactants. Unlike other ionic surfactants, which are somewhat toxic, these show excellent biocompatibility. This is most likely due to the fact that lecithin is obtained naturally from soybean or egg, which contains diacylphosphatidylcholine as the major constituent [179]. Another important class of zwitterionic surfactants to note is the betaines, such as alkylbetaines, amidoalkylbetaines, and heterocyclic betaines [184].

Surfactant Mixtures and Co-Surfactants

More often than not, one surfactant, whether nonionic or ionic, is not sufficient to form a microemulsion or does not result in an optimal microemulsion-forming region. Combinations of surfactants or sometimes co-surfactants are required for the optimal formation of a microemulsion. The term "co-surfactant" can describe any component that aids the primary surfactant in microemulsion formulation. "Co-surfactant" can refer to a second surfactant being used, but may also refer to a low-molecular-weight amphiphile, such as an alcohol.

Two different nonionic surfactants can be mixed together. Mixing a more lipophilic nonionic surfactant with a more hydrophilic nonionic surfactant can result in the exact

HLB needed to form a microemulsion. The two surfactants can be mixed in varying ratios to determine the ideal combination of the two, which results in the largest microemulsion-forming region. Mixtures of nonionic surfactants can be seen in commercial products and can sometimes be regarded as a single component (a pseudo- component) in the microemulsion system.

Ionic surfactants can be combined with nonionic surfactants, or higher molecular weight ethoxylated alcohols. These mixtures have synergistic effects, which allow them to be applied to many things. The most popular advantage to these mixtures is the fact that they result in temperature insensitive microemulsions. Generally, ionic and nonionic surfactants react oppositely with increasing temperature. Ionic surfactants show a hydrophilic shift with increasing temperature, while nonionic surfactants exhibit a lipophilic shift. Therefore, when mixed together in a particular ratio, the two will cancel each other out, resulting in a temperature insensitive microemulsion formulation.

Frequently, single chain surfactants are not able to reduce the surface tension to the ultra- low levels required for microemulsion formulation. Short and medium chain alcohols, such as butanol, pentanol, ethanol, isopropanol, or propylene glycol, are commonly added as "co-surfactants" [179]. These co-

surfactants help to further reduce the surface tension and fluidize the surfactant film, which increases the entropy of the system leading to its thermodynamic stability. Co-surfactants increase the flexibility of the surfactant film around the microemulsion droplet [179]. The co-surfactant molecules distribute themselves between the oil, water, and oil/water interface. The relatively small co-surfactant molecules ultimately get mixed in with the surfactant molecules at the oil/water interface. This releases the bending stress and allows for easier dispersion [185]. These alcohols increase the fluidity of the hydrocarbon tails of the surfactant. This allows greater penetration of the oil into the surfactant monolayer. As the chain length of the alcohol increases, the flexibility of the film decreases. Alkanols introduce more disorder into the interfacial film since their chain length is much different from the surfactant molecules. Molecules move laterally as the interfacial film spontaneously fluctuates. As an added benefit, alkanols added to ionic surfactants serve to reduce the repulsive forces between the charged head groups of the surfactant molecules. In the case of lecithin microemulsions, an alcohol must be added as a co-surfactant to disrupt the lamellar structures, which characterize its biological behavior allowing for the formation of a microemulsion [179].

Incorporation of co-surfactants can expand the microemulsion-forming region, but this may come at a cost. The requirement of a medium-chain alcohol as a co-surfactant may cause other problems. Many of these alcohols can be irritating to the biological system, especially with chronic use. There are significant toxicity issues with these chemicals, which may prevent microemulsions containing them from being used pharmaceutically [179]. Solubility of the alcohols in microemulsion formulations becomes an issue as well. Most alcohols tend to be more soluble in the aqueous phase of o/w systems than the primary surfactant. Because of this, as the system is diluted, the co- surfactant partitions more in the water-phase and reduces the amount of co-surfactant present at the interface. This destabilizes the droplets, and ultimately the microemulsion system itself. Short chain amines and alkanoic acids are also suitable co-surfactants, but these prove to have similar toxicity issues to the alcohols [179].

Advantages and Disadvantages

As with all things, microemulsions have advantages and disadvantages. Because of the ease of microemulsion preparation, drugs that are thermo-labile are easily incorporated without the risk of degradation [186]. In drug delivery, microemulsions ultimately increase the surface area of drugs, which improves their solubilization and permeation behavior. They are shown to increase solubility and bioavailability of Class II and IV drugs of the biopharmaceutical drug classification system. Class II drugs have high solubility, but low permeability and Class IV drugs have low solubility and low permeability. Plasma concentration profiles and drug bioavailability have been shown to be more reproducible in microemulsion formulations [179]. The rate of penetration of drug is much faster

from microemulsion systems than from other drug delivery vehicles, while having controlled drug release rates, slow degradation, and target specificity [186]. Microemulsions have a higher solubilization capacity for both hydrophilic and hydrophobic compounds than simple micellar solutions. Because of their thermodynamic stability, they are more favorable than regular emulsions or suspensions since microemulsions can be manufactured with very little energy input and have a long shelf life.

Microemulsions have some disadvantages as well. Formation of microemulsions generally requires large amounts of surfactants and/or co-surfactants. All of these at high concentrations are generally irritating, if not slightly toxic, to the biological system [186]. Many outside factors, such as temperature and pH, influence the stability of microemulsions as well. The effects of these factors must be examined for each potential formulation.

3.2 Omega-3 fish oils Micro Emulsions and challenges

The most widely used delivery systems for incorporating omega-3 oils into foods and beverages are bulk oils, emulsions, and powders (76). These powders are typically formed by spray drying emulsions. Microencapsulation has proved to be a popular way of creating powdered omega-3 that can be incorporated into a variety of food products including baked goods, spreads, and fruit beverages . However, this technology typically only delivers relatively small levels of bioactive lipids since powders usually only contain around 1 to 30% omega-3 FAs [187].

A number of obstacles must be overcome before omega-3 fortified microemulsions can be successfully incorporated into commercial food products, such as their susceptibility to lipid oxidation, ensuring the physical stability of the system, delivering a nutritionally beneficial quantity of bioactive in a bioavailable form, and providing a palatable product that is acceptable to consumers. A number of these challenges are discussed in more detail in the remainder of this section.

Lipid oxidation in food products causes multiple problems that impact shelf-life, safety, nutritional value, functionality, and flavor [188]. Oxidation is readily noticed by consumers because the products of the reaction cause undesirable sensory attributes in food products at very low levels . Oxidation is the reaction of unsaturated FAs free radicals and oxygen (Figure 19) and occurs in three stages: initiation/induction, propagation, and termination [188] . The most common mechanism for oxidation in emulsions is the reaction of free radicals with unsaturated lipids leading to the formation of lipid radicals. These lipid radicals react with oxygen and other lipids, thus beginning the chain reaction

(propagation) stage of lipid oxidation [189]. Before oxidation occurs, there is a lag phase, which is the phase that food processors attempt to extend through means of storage in cooler temperatures, decreased oxygen exposure, and addition of antioxidants [189]. Once the initiation phase has begun, the rate of oxidation increases exponentially and the food is spoiled.

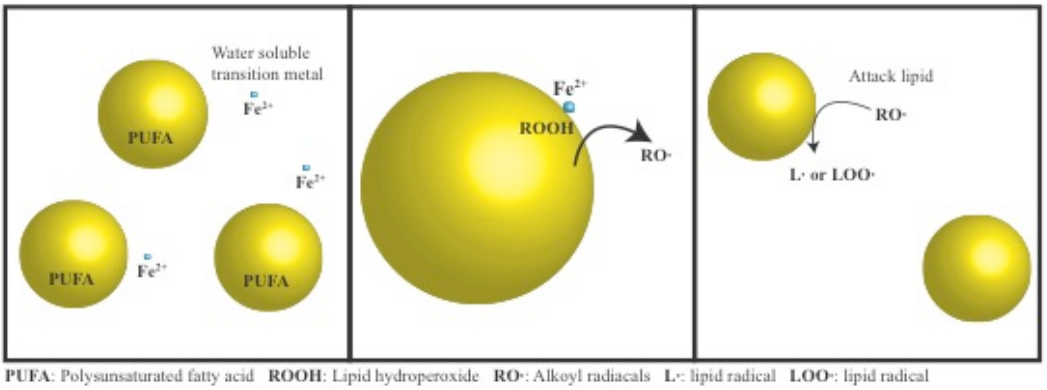


FIG 19: Proposed mechanism of lipid oxidation in an oil-in-water emulsion or microemulsion.

Lipid oxidation is promoted by exposure of unsaturated lipids to air, light, heat, and irradiation [188]. Many factors contribute to an emulsion-based delivery system's susceptibility to oxidation including the composition, structure and organization of the oil, water and interfacial phases, as well as the type, amount, and location of any antioxidants present [190]. FO microemulsions are particularly susceptible to lipid oxidation for a number of reasons: high degree of lipid unsaturation; high surface area of exposed lipids; and greater light penetration [190]. Indeed, experimental studies have shown that lipid oxidation is faster in protein-stabilized microemulsions than in conventional emulsions with similar compositions, which was attributed to the higher lipid surface area. Consequently, it may be necessary to take additional steps to stabilize omega-3 oils encapsulated within microemulsions when compared to conventional emulsions.

Oxidation in microemulsions can be partially managed by controlling their physicochemical characteristics. Surfactants can influence the droplet charge, thickness, and permeability, all of which control the ability of pro-oxidants, free radicals, and oxygen to interact with the lipids in the droplets [190]. Several studies have shown that anionic surfactants attract cationic transition metals while cationic surfactants repulse them thereby decreasing the rate of oxidation [190]. In addition, the interfacial layer of an emulsion can form a physical (steric) barrier against the aqueous phase of a system that contains pro-oxidants [190].

To prevent oxidation in food systems, radical scavenging and metal chelation are the main antioxidant strategies [190]. Free radical scavengers react with free radicals before they can react with unsaturated

FAs, and their effectiveness depends on their ability to donate a hydrogen atom to the free radical. Studies of the chemical degradation of β- carotene in emulsions (another polyunsaturated bioactive lipid) have shown that the rate of oxidation depends on system conditions (such as pH, ionic strength, temperature, droplet size, and emulsifier type) and can be inhibited by adding appropriate antioxidants [191]. The addition of antioxidants has also been found to improve the stability of citral oil in emulsions [192]. Similar factors are likely to affect the rate of omega-3 oxidation in emulsions. Vitamin C, E and A can act as antioxidants through the means of radical and oxygen scavenging. They have been tested for their ability to inhibit oxidation of PUFA methyl esters in oil-in-water emulsions [193].

With the growing use of emulsion-based delivery systems for human consumption, it is important to evaluate the gastrointestinal fate of the systems to ensure that there are no adverse health effects, and that the bioactive being delivered is indeed being absorbed into the body [194]. *In vitro* and *in vivo* digestion models have become instrumental in undertaking this kind of evaluation [195].
Bioaccessibility is an important marker used in these studies that describes the fraction of an ingested compound (the bioactive) that is transferred into a mixed micelle after lipid digestion [196].
An ingested emulsion will pass through the mouth and stomach before reaching the small intestine where lipid absorption normally occurs [197]. The size, composition, and surface characteristics of the lipid droplets within a microemulsion may change appreciably when they are exposed to gastrointestinal conditions. Upon entering the small intestine, lipase adsorbs to the surfaces of emulsified fats and coverts triacylglycerols into monoacyglycerols and free fatty acids (FFA). These FAs are then incorporated into mixed micelles, travel through the mucus layer, and are absorbed by epithelium cells. The bioavailability of encapsulated fatty acids may be inhibited if the ability of the lipase to adsorb to the surface of lipid droplets and hydrolyze the triglycerides is prevented. The type and amounts of surfactants in a microemulsion may therefore impact the rate and extent of lipid digestion and FFA release. For example, corn oil nanoemulsions made using high-energy methods experienced a lag period before FFA release that ranged from 5 to 20 minutes as the mean droplet radius increased [198]. This was a result of the lipase not being able to adsorb to the surface of the droplets due to the presence of excess surfactant that competed for the droplet surfaces. In such emulsions, 61-71% of the FFAs were released with higher amounts of FFA being release as the particle radius decreased. The obstruction of lipase as a result of high surfactant concentrations was also seen in medium chain triglyceride nanoemulsions containing vitamin E acetate made from both high and low energy methods [199]. In this study, both the high and low energy emulsions had

comparable particle sizes and similar behaviors throughout the *in vitro* digestion and both released similar amounts of FFA.

Surfactants can also impact the rate of lipid digestion based on their molecular and physicochemical characteristics. A study by Speranza et al. evaluated the effect of nonionic and anionic surfactants with a range of HLB numbers on the bioaccessibility of lipids (trioctanoyl glycerol) in emulsions using an *in vitro* digestion model [200]. The results showed that an increasing HLB number increased the lag time in the jejunum and decreased the rate of lipolysis. In contrast, increasing the length of the aliphatic chain decreased the lag time in the jejunum, but increased the rate of lipolysis in the small intestine.

After FFA and bioactives are liberated from the lipid droplets, they form mixed micelles that travel through the mucus layer, and are then absorbed by the intestinal epithelial cells. When conventional FO emulsions were compared with FO nanoemulsions, the nanoemulsions had a significantly higher percentage of lipid absorbed compared to the conventional emulsions, which was attributed to their smaller particle size [201]. A recent study showed that the bioaccessibility of an oil- soluble bioactive component (vitamin E acetate) was higher in nanoemulsions prepared using a low-energy method (emulsion phase inversion) than in those prepared using a high-energy method (microfluidization). It was suggested that the high levels of surfactant used in the low-energy method may have increased the amount of bioactive incorporated into the mixed micelles. The surfactant characteristics can also impact FFA absorption. An increasing surfactant HLB has been reported to increase the bioaccessibility of FFAs in the small intestine.

Lastly, the absorption of FOs from ingested foods is important when developing functional food systems. Researchers investigated the absorption of FO in capsules *versus* microencapsulated FO incorporated into a milk shake [202]. Both treatments resulted in similar increases of EPA and DHA in blood plasma. Another study looked at yogurt as a carrier product for algal oil emulsions (mean droplet size 258 nm) *versus* bulk oil. In this study, both the emulsion and bulk oil increased DHA levels in blood lipids however, DHA from the emulsion was more bioavailable than the bulk oil during the first four hours of digestion. Both of these studies support the use of microencapsulated or emulsified FO in food products and provide an alternative way for consumers to supplement their EPA and DHA intake without swallowing a large pill. The properties of a food system that accompanies the FO also has importance. When supplements were consumed with a higher fat meal compared to a lower fat meal, more long chain omega-3 PUFA were available, possibly due to the higher fat content stimulating more digestive enzymes and more mixed micelles. This again supports the use of functional foods to incorporate omega-3 FA and increase the absorption of the fats as an alternative to supplements.

Findings showed that dietary DHA supplementation could rapidly recover the DHA concentration of tissues in n-3 deficient mice during the childhood the higher hepatic DHA levels were found when DHA supplementation was added with α-GPC (alpha glyceryl phosphoryl choline) after short-term DHA supplementation for 4 days. In addition, DHA-TG+α-GPC exhibited the greater effect on DHA accumulation in cerebral cortex and erythrocytes [203].

Some consumers must find alternative sources of omega-3 FAs because they do not like the flavor or texture of fish or seafood. High quality refined FOs have little to no flavor. This is unlike oils used in some dietary supplement that are low quality and have strong fishy flavors. Some consumers avoid soft gel capsules of FO supplements because of the reflux of FO resulting in "fish burps" [204]. This is caused by the formation of a layer of the FO on top of the stomach contents because the oils have a lower density than the gastric juices. By using a FO microemulsions, consumers can receive the benefits of EPA and DHA in a form other than seafood. In addition, emulsions can be designed to be resistant to coalescence and creaming within gastric environments by selecting appropriate emulsifiers so that the oil will not form a layer of oil on the top of the stomach contents and cause reflux issues [205].

Studies disagree about which types of food as bioactive component, such as omega-3 oils, should be added for maximum consumer interest. In a study by Ares and Gámbaro consumers were more accepting of a functional food when the carrier food was perceived as being healthy [206]. In a separate study by Bech-Larsen and Grunert, it was concluded that functional foods with a healthier base food were perceived as healthier compared to functional foods with an unhealthy base food, however this study also stated that consumers rationalized the enrichment of less healthy foods better than that of already healthy foods [207]. Some consumers have concerns about unhealthy foods that have been fortified because they may now be perceived as a health food by others when they are not [208].

Regardless of the carrier product, it is important to the consumer that the bioactive ingredient and base food are compatible; this is a stronger driving force for the purchasing of functional food products compared to health benefits and attitude towards functional foods. For example, products where FO appears to be a more natural fit such as fish balls, rye bread, and tuna salad were expected to receive more positive attention by consumers [208]. Another characteristic of FO enriched foods that should be considered when choosing an appropriate food carrier and in the product formulation is the sweetness profile. Participants in a study evaluating the acceptance of FO fortified foods were put off by sweet products such as yogurt drinks and sports bars having the addition of FO [208] . In

a separate study, women between the ages of 40 and 60 years did not accept the addition of sweeteners into a functional food and would rather consume a more natural product [209].

It is suggested that the use of health claims on functional food labels will have a positive impact on the consumer's view of the healthfulness of that food [207]. The source of omega-3 fatty acids used in the fortification of foods can affect the cost of the products but also their health benefits. ALA omega-3s may give a cleaner label because they are from plant sources along with a lower price for consumers however, the conversion of ALA to LC-PUFA is quite low, decreasing its actual health benefits. The FDA health claim for EPA and DHA containing foods can aid in the marketing and advertising for qualifying products while differentiating them from products that only provide ALA.

Finally, sensory aspects also play a key role in consumer acceptance of foods. Few studies have researched the effect of nanoemulsions on the sensory properties of enriched foods. Dairy products have been the main focus of these studies. One study evaluated the fishy off flavor intensity of strawberry yogurt containing emulsified omega- 3 oils after 14 days storage. This study found no significant difference between the control and fortified yogurt samples amongst an untrained consumer panel. Another study evaluated a strawberry drinking yogurt fortified with bulk algae oil and algae oil nanoemulsion for smell, appearance, flavor, texture, consistency, aftertaste, and overall acceptability [210]. Consumers were able to identify a sensory difference between yogurts fortified with either bulk oil or nanoemulsions in a triangular test. However, no statistically significant differences were found between the nanoemulsion-fortified, bulk oil-fortified, and unfortified yogurts in terms of their consistency and appearance. The sensory properties of cheese fortified with bulk FO or FO emulsions have also been evaluated [211]. Fishy off flavor was dependent on the concentration of FO in the sample and was more easily detected in the bulk oil-fortified samples compared to the emulsion-fortified samples. Clearly, more research should be conducted to evaluate the sensory aspects of foods fortified with emulsions to better understand their effect on consumer acceptance.

Materials and Methods

1. Materials and Instruments

For the lipidomic analyses chloroform, methanol, n-hexane (HPLC grade, sodium sulphate anhydrous (Na2SO4) and Potassium hydroxide (KOH) were purchased from Carlo Erba (Milan, Italy). All FAMEs used as standard references for GC analysis were purchased from Sigma Aldrich or Fluka (Switzerland) and used without further purification.
The DHA emulsion was prepared with Omega-3 Fish oil (vivOmega 0370) purchased fron VARIATI (Monza, Italy), Vitamin C (99,9%), Medium chain triglycerides (MCT), Vitamin E (alfa tocopherol 70 % in sunflower oil) , alpha lipoic acid (ALA), citric acid, potassium sorbate, peach aroma and lemon essential oil were purchased from Farmalabor (Milan, Italy), alpha glyceryl phosphoryl choline (L-GPC, PHOSAL ®) was purchased from Lipoid (Ludwigshafen, Germany) and Astaxantin (Astazine ®) was purchased from BGG (Lugano, Switzerland) .
Membrane cells incorporation was measured using the membrane fatty acid-based lipidome analysis of HELA, HEK and DAOY cell lines, the cultures being carried out in the Department of Pharmacology, University of Bologna, IT with the collaboration of the group of Prof. Spampinato.
Fetal Bovine Serum (FBS), L-Glutamine and penicillin-streptomycin solution were purchased from Sigma-Aldrich (San Louis, MO, USA). Trypsin/EDTA was from BioWhittaker Europe, Verviers, Belgium. Flasks and plates were from Falcon (Franklin Lakes, NJ, USA). All the other cell culture reagents were from Sigma-Aldrich. The water used was prepared by the device Milli-Q (Millipore, Milford, MA USA) and was acquired at a resistance value of 18 MΩ at the source. Silica TLC plates were purchased from Macherey-Nagel.

Two instruments were used to obtain the results described in this thesis:
DLS: Dynamic Light Scattering, Malvern Instruments Series NanoZS with a detection angle of 173◦, Malvern Instruments, Malvern, UK

GAS CHROMATOGRAPH (GC): Agilent 789B0 Series gas chromatography apparatus using ChemStation software (Agilent 6850, Milan, IT). The instrument has a flame ionization detector (FID) that requires air (450 mL/min) and hydrogen (40 mL/min) and is maintained at a temperature of 230 °C.

2. Methods

2.1 Emulsion Preparation

The emulsions were prepared by a mixer (Bosh). Water phase (ascorbic acids wt%, citric acid, sodium benzoate) was mixed with L- α-GPC, sorbitol , lipoic acids and flavours for 10 min at 750 rpm. The oil phase was prepared stirring for 10 min at 750 rpm Fish oils (5 wt%) alfa-tocopherol and Astaxantine. Oils and aqueous phases were combined and mixed. The emulsions were mixed for 30 min at 750 rpm.

Each formulation was produced in triplicate. All samples were stored in amber glass bottles at 20 °C, or 4°C in the dark for 28 days for stability evaluation.

The analytical techniques for the detection and quantification of lipids are characterized by high selectivity, that is the ability to determine the analyte in the presence of interferents in the matrix.

The lipid analysis of a sample usually involves some common key steps:

- extraction of the lipid fraction from the starting matrix (emulsion);
- isolation of the lipid fractions of interest;
- derivatization of the lipid components;
- identification and quantification of FAs.

The accuracy of the analysis is influenced by the effectiveness of each single step. For this reason, the analytical method of extraction should be developed taking into account the following requirements:

ability to inhibit the action of enzymes such as lipases and phospholipases, in order to avoid lipid degradation during the analysis;

ability to limit oxidative and isomerization phenomena of the double bonds during extraction (generally induced by high temperatures and unsuitable solvents);

ability to extract all the constituents of the lipid fraction;

ability to separate lipids from other low polarity components (apolar interferents) that could compromise the quality of the analysis.

2.2 Lipid extraction

Lipid extraction permits to isolate lipids from biological matrices and remove any sources of interference, such as proteins and sugars. Lipids are usually characterized by hydrophobic properties that make them immiscible with water and soluble in non polar (organic) solvents. However, due to the broad variety of lipids, some classes of lipids, such as phospholipids, have amphiphilic properties, that confer them greater polarity.

For this reason, the most efficient lipid extraction method is the liquid-liquid extraction proposed by Folch in 1957, which suggested the use of a chloroform/methanol mixture (2:1 v/v) for quantitative lipid isolation [212]. Even if other effective mixtures were identified, aimed at reducing the solvent toxicity and increasing selectivity, Folch method remains the most commonly used procedure for the extraction of lipids from biological matrices.

2.3 Thin Layer Chromatography (TLC)

After the extraction step, thin layer chromatography (TLC) can be used to verify in a fast and relatively unexpensive way the efficiency of the extraction method. Usually, it is particularly suitable for carrying out qualitative or semi-quantitative evaluations as well as to follow the progress of a chemical reaction.

Like all chromatographic techniques, TLC is based on the different distribution of similar substances between a stationary phase and a mobile phase, depending on the affinity of each substance with the substrates. Molecules with high affinity for the mobile phase will migrate faster along the plate, while molecules with high affinity for the stationary phase will stuck close to the baseline, without significally migrating. The TLC plate is usually coated with a suitable adsorbing material, such as silice gel or alumina, that constitutes the stationary phase. The mobile phase is a solvent (or a mixture of solvents) that is characterized by a low affinity with the stationary phase and a good ability to separate the components of interest. Depending on the polarity of the lipid families present in the sample, different mobile phases can be employed.

The procedure consists on the deposition of a small amount of the lipid sample on the TLC plate, that is then immersed in a chamber saturated with the appropriate mobile phase. Over time, the solvent moves up the TLC plate due to capillary forces and separates different lipid fractions based on their affinity for the absorbing material. Standard reference substances are usually spotted beside the lipid mixture to allow recognition and assignment of the lipid components.

Since most lipids are colorless and TLC spots cannot be seen, stains can be applied at the end of the separation by spraying - or by dipping the plate into - a staining solution, to form colored compounds that can be identified by direct observation or UV analysis.

For these set of experiment, cerium ammonium molibdate (CAM) was chosen as staining technique, due to its sensitivity and wide applicability. CAM can be prepared by dissolving 1.5 g of phosphomolybdic acid and 1.0 g of cerium (IV) sulfate in a mixture of 6 ml of sulfuric acid (H_2SO_4) and 94 ml of distilled water. After exposure to heating conditions, Mo^{5+} and Mo^{4+} compounds are formed and green to blue colored spots on a yellow background appear on the plate.

Each substance has a specific retention factor (R_f), corresponding to the distance of the spot from the origin compared with the distance covered by the solvent (solvent front). By comparing the distance reached by the experimental spots with that of standards of known composition, it is possible to identify the lipids present in the original mixture (Figure 20).

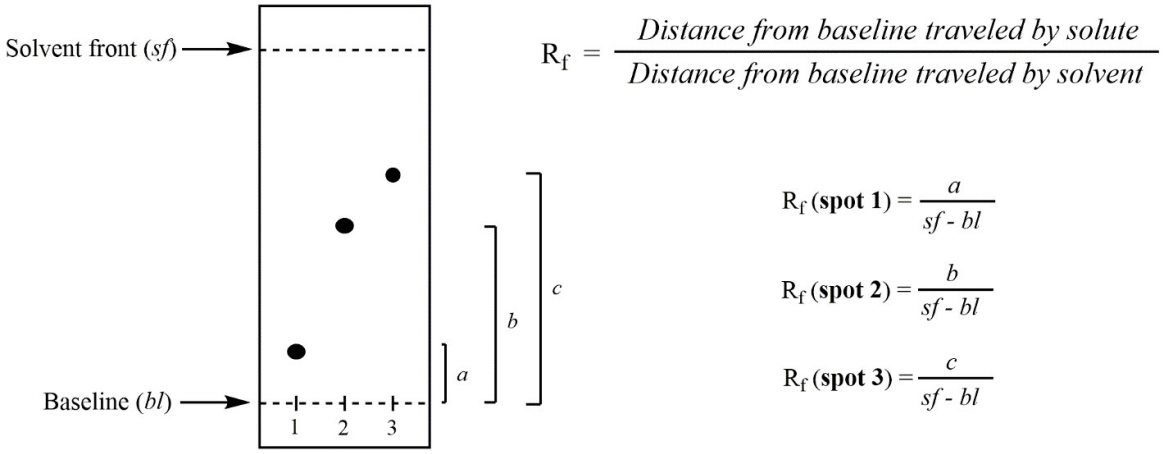


FIG 20: Schematic representation of a TLC plate and R_f definition.

2.4 Gas Chromatography (GC)

The step that follows lipid extraction is the fatty acid derivatization to more volatile compounds, using the transesterification reaction that leads to fatty acid methyl ester (FAME) derivatives. FAMEs can be analyzed by gas chromatography (GC), that is the election technique for lipid separation and quantification. The derivatization step is necessary since most lipids have low volatility and some tendency to degrade at high temperatures.

The transesterification procedure to FAMEs can take place with both acidic or basic reagents as catalysts. The most efficient derivatization method to obtain FAMEs is the transesterification reaction carried out in a basic environment [213]. The conditions usually require anhydrous methanol (CH_3OH) in the presence of a basic catalyst, usually potassium hydroxide (KOH), which facilitates the exchange between methanol and glycerol of the O-acyl lipids, such as triglycerides and phospholipids (Figure 21).

In this step, not only the base but also the molarity and type of solvent used become determinative for the quantitative success of the reaction. In basic conditions, the reaction takes place in a very short time without inducing isomerization and migration phenomena, processes that easily take place in acidic environment. Attention must be paid to exclude the presence of water in the reaction environment and to prevent the occurrence of hydrolysis and FFA formation. Water is used to quench the reaction, since it neutralizes the anions present, and FAMEs can be extracted with hexane and, once anhydrified, can be injected in the GC.

GC equipment (Figure 22) consists of a sample injection system, connected to a capillary column that is flushed with the carrier gas in a thermostated chamber (oven). The column leads to a detector that is connected to an external unit equipped with software for data processing. In gas chromatography, the mobile phase is a carrier gas, usually an inert gas such as helium or an unreactive gas such as nitrogen. The sample is heated in the injection chamber to volatilize the FAMEs and then carried into the separating column by a heated carrier gas.

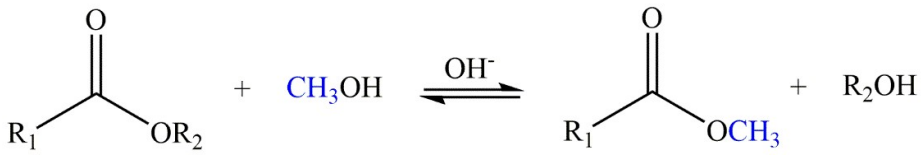


FIG 21: Base-catalysed transesterification mechanism of an acyl chain to its methyl ester derivative.

To inject a liquid phase sample into a capillary column, spit or splitless modes can be used. In the split mode, 1 µL of the sample is introduced into the injector, but only a small percentage of steam generated within the liner enters in the column, while the remaining part is removed though the operation of a valve. This mode guarantees high resolution separations thanks to the high flow rate at the split point. In the case of trace compound analyses, however, the splitless mode is more indicated, which involves the processing of the full injected sample in the column.

FAMEs in the mixture can be separated because of their differing affinities for the matrix in the column. The stronger the affinity between a specific molecule and the matrix the slower it passes

through the column. After being separated by the column, each molecule passes by a suitable detector that allows its quantification.

For the GC analyses reported in this thesis, a gas chromatograph (GC) equipped with a 60 meters capillary column in fused silica having a stationary phase consisting of (50%-cyanopropyl)-methylpolysiloxane (DB-23, Agilent, USA), was used. The GC is connected to a flame ionization detector (FID). The operating mechanism of FID provides the detection of ions formed by organic compound combustion on a hydrogen flame, that is proportional to the concentration of organic species in the sample gas stream. The molar response of hydrocarbons is usually equal to the number of carbon atoms in their molecule, while oxygenated lipids and other species that contain heteroatoms tend to have a lower response factor.

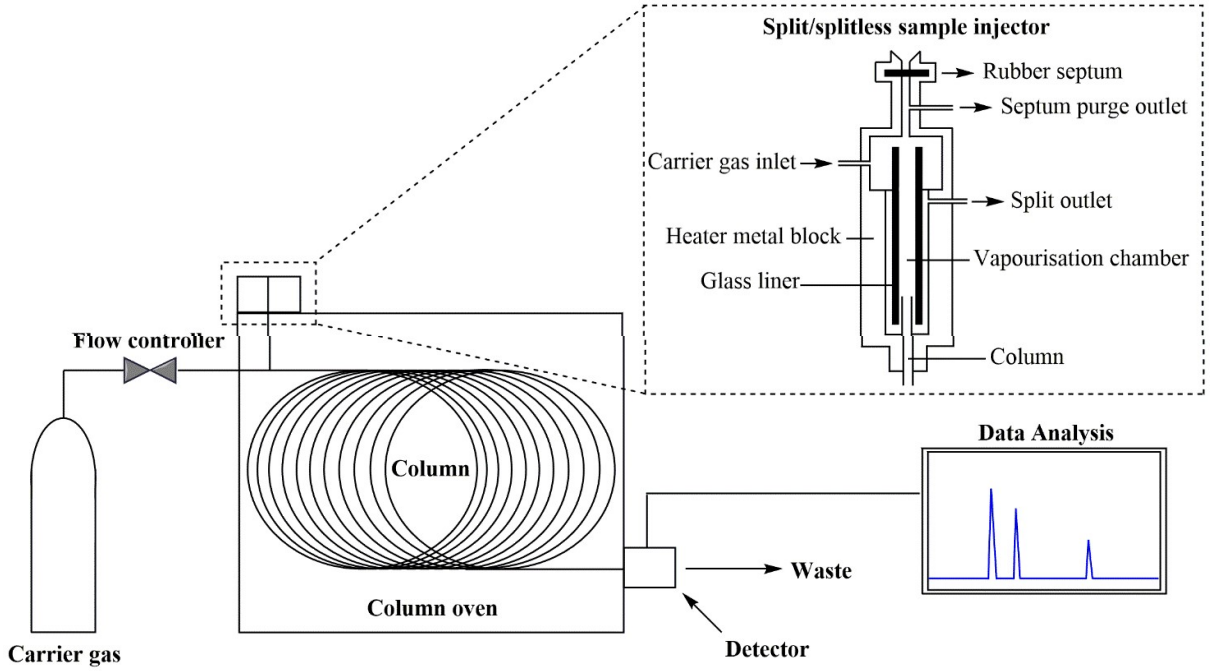


FIG 22: Schematic representation of a gas chromatogram equipped with a spit/splitless sample injector.

For more details regarding gas carrier, volume of injection, oven temperature program and operating pressures, please refer to the experimental sections of each chapter.

The specificities of DB-23 column, including the length and the medium polarity of the stationary phase, combined with the oven temperature set up, guarantee the selectivity and efficiency required for the resolution of complex FAME mixture and achieve the separation of a high number of geometrical isomers.

With the conditions set up in our laboratory, elution order depends on the number of carbon atoms and the number of double bonds in the acyl chain. For example, the retention time of FAMEs

increases as the number of carbons increases, being short chain fatty acids the first ones to elute and long chain fatty acids the last ones (14:0 < 16:0 < 18:0 < 20:0 < 22:0). When chains differ in the number or in the position of unsaturated bonds rather than in the chain length, SFAs are usually the first ones to elute, while MUFAs and PUFAs strongly interact with the stationary phase and are retained with increasing strength inside the column (18:0 < 18:1 < 18:2 < 18:3).

Considering the position of the double bonds, usually FAMEs with the unsaturation closer to the carboxylic group are eluted first (9c,18:1 < 11c,18:1), and the same happens when the double bond has trans geometry (9t,18:1 < 11t,18:1). In analogy with saturated FAMEs, when the only difference is the configuration of the double bond, trans isomers are less retained compared to the cis analogues (9t,18:1 < 9c,18:1).

However, these should only be considered general indications, because for FAMEs with more than 18 carbon atoms, the separation of trans isomers does not comply with these elution criteria. The unpredictable elution of PUFA methyl esters with more than 18 carbons reinforces the need of molecular reference libraries of standards for the precise interpretation of GC chromatograms. The most common FAMEs with the cis geometry are commercially available. However, for the mono-trans isomers of our interest, such as ARA [8] and EPA [9], it was necessary to build up synthetic methods for the full characterization and the correct allocation of the peaks.

GC presents many advantages, since it requires small samples (typically 1-2 μL) and is characterized by high resolution and high sensitivity for effect of the capillary columns. Depending on the length of the column and the set-up of the oven ramp, the analyses can require longer analysis times.

FAME were analyzed by gas chromatography (Agilent 780B, Milan) equipped with a 60 m x 0.25 mm x 0.25 μm (50%cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA), a flame ionization detector (FID), with injector temperature at 230 °C and split injection 50:1. Oven temperature started from 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195°C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, and held for 10 min. A constant pressure mode (29 psi) with helium as a carrier gas was used. Methyl esters were identified by comparison with the retention times of commercially available standards or trans fatty acid references. The list of the examined FAME (corresponding to chromatographic peak areas >97%) in membrane PL are reported as % relative percentages of GC area.

The detection and quantitation limit were determined by comparing measured signals from the chromatograms of samples with known low concentrations of analyte with those of blank samples. For the detection limit, the acceptance criterion was a signal/noise ratio of minimum 3:1, while for the quantitation limit the acceptance criterion was a signal/noise ratio of minimum 10:1.

The external standardization method was applied for the GC quantification and it was performed within the linear range of response. Calibration curves (GC area vs FA concentration) were obtained using standard solutions of known concentration for each FA moiety. Calibration curves were considered linear when the coefficient factor R^2 was equal to or greater than 0.995. Representative calibration curves are depicted below:

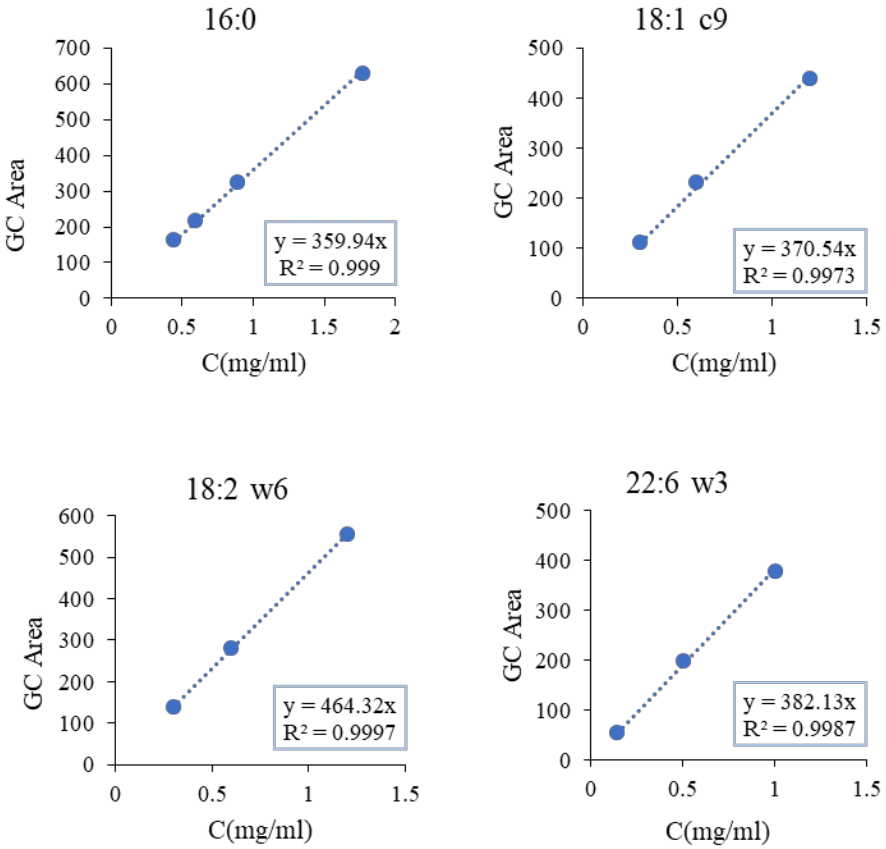


FIG 23: Calibration curves of four representative fatty acid methyl esters (FAME): (A) the saturated methyl palmitate (16:0); (B) the monounsaturated methyl oleate (18:1 c9); (C) the ω6 polyunsaturated methyl linoleate (18:2 ω6) and (D) the ω3 polyunsaturated methyl-(22:6 ω3).

Analyte's concentration was calculated by the following expression:

$$Cx = Cstd \; x \; Ax \; Astd$$

where,

Cx: the concentration of the x-FA in the sample

Cstd: the concentration of the x-FA in the standard solution Ax: the area of the x-FA from the sample chromatogram Astd: the area of the x-FA from the standard chromatogram.

2.5 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 7.02 software for Windows, GraphPad Software, La Jolla California USA. All measurements were performed in replicates and the data were expressed as the mean ± standard deviation (SD). Statistical significance was based on 95% confidence intervals ($p \leq 0.05$).

Comparison of two groups

For the comparison of two groups, an unpaired Student's t-test was employed to analyze the data.

Comparison of three or more groups

For the comparison of three or more groups, data were analyzed by one-way analysis of variance ANOVA test combined with BONFERRONI's test.

2.6 Dynamic Light Scattering

One of the most important characterizations of microemulsions is particle size. This can be determined with the use of dynamic light scattering (DLS). Dynamic light scattering is also known as photon correlation spectroscopy. Small samples of solution (less than 1mL) are needed for the measurement and measurements are made rapidly which are a few of the advantages of this type of particle size analyzer [214]. In simplest terms, a laser is passed through a filter directly onto the scattering medium. The scattered light is then detected by the detector and translated to an autocorrelator [215]. A schematic of DLS can be seen in Figure 24.

An important concept related to DLS is the utilization of Brownian motion of particles in a medium. Particles are known to diffuse in a solution spontaneously. Colloidal particles in a solution move randomly and erratically as a result of inter-particle collisions. This erratic movement is known as Brownian motion. DLS uses this phenomenon in estimating the particle size. As the particles randomly diffuse in solution, the size of the particles can ultimately be determined by how rapidly they diffuse through the medium.

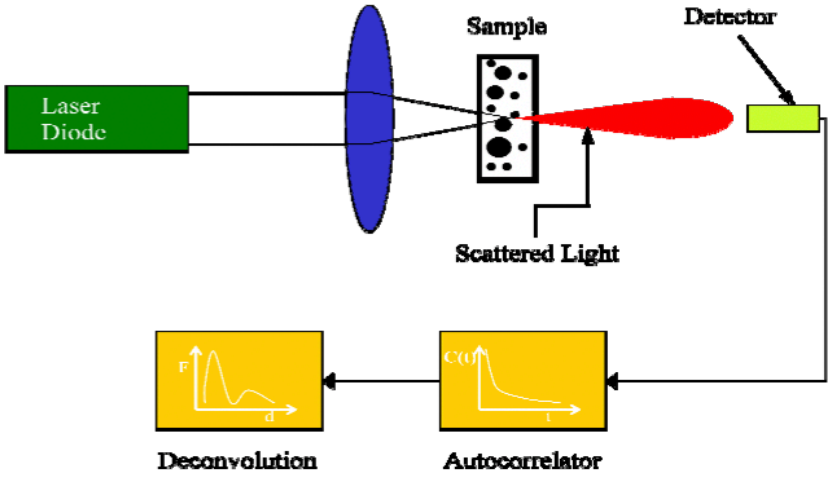


FIG 24: Schematic of dynamic light scattering used to measure particle size

Dynamic light scattering (DLS) experiments were performed to determine particle size of the microemulsions and the stability during the time. Microemulsions were stored at 20 °C for 2 months and measured every week.

Before each measurement the emulsions were diluted 1:50 with MilliQ water. Microemulsion samples were transferred into 6x50 mm DLS tubes and then placed in the Dynamic Light Scattering, Malvern Instruments Series NanoZS with a detection angle of 173◦, Malvern Instruments, Malvern, UK) and particle size determined. Four cycles were run at 2 minutes per cycle for each sample, with a channel width of 5.5 µSec, temperature of 23°C, and 90° scattering angle. Mean volume weighted diameter was then calculated from the four runs for each sample.

2.7 Cell cultures

The incorporation of DHA into plasma membrane was assayed on HELA, DAOY and HEK cells that derived from a human ovarian cancer, medulloblastoma and Human embryonic kidney respectively. Cells were cultured as a monolayer at 37 °C in a humidified atmosphere at 5% CO_2 in complete medium (RPMI 1640 supplemented with 10% heat- inactivated FBS, 2 mM L-Glutamine, 100 units/mL Penicillin, 0.1 mg/mL Streptomycin). Cultures were maintained in the log phase of growth with a viability >95%. The viability was checked before each experiment.

Before any treatment cells were incubated for 24 hours.

In every experiments the cell lines were treated with a concentration of oil or emulsion of 0.5 g / L because this is believed to be the suitable concentration both to promote normal cell proliferation and a response to treatment.

Furthermore, this concentration does not lead to differences in the pH and osmolarity of the culture medium [216].
The experiment was conducted by treating the cells for 24 h with 15 μM of the full composition of our microemulsion, 1.5 mM of pure fish oil and 15 μM of an emulsion O/A containing the same concentration of fish oil as present in the microemulsion but without antioxidants and carriers. After 24 h, the cell pellet was first washed with PBS and centrifuged, for complete removal of the medium. Finally, we proceeded with the addition of water to promote cell lysis and the isolation of the membranes only by means of a 15-minute centrifugation at 14,000 rpm and 4 ° C [39] [204].

The analysis was carried out through the use of the automatized Robot (LRN-R1) for the extraction and lipid transesterification for greater repeatability.
The experiment was conducted in triplicate for each type of treatment and control.

2.8 Erythrocyte membrane fatty acid analysis

A first test for the Human bioavailability was made supplementing 5 healthy adults volunteers (3 Males and 2 Females, mean age 27 ± 0.8 years).
The fatty acid composition of mature RBC membrane phospholipids was obtained from blood samples (approximately 2 mL) collected in vacutainer tubes containing ethylenediaminetetraacetic acid (EDTA). Samples were stored at 4ºC until analysis. Blood work-up for lipid extraction and lipid transesterification to fatty acid methyl esters (FAMEs) was performed using an automated protocol, which included a selection of mature RBC, widely reported in the literature [86] [101]. The method has been certified by the National Body of Accreditation (Accredia) to be compliant with the ISO:17025. Briefly, the whole blood in EDTA was centrifuged (4000 rpm for 5 min at 4ºC), and the samples entered an automated procedure for mature RBC selection. The mature cell fraction was isolated based on the higher density of the aged cells. All the subsequent steps for the cell lysis, isolation of the membrane pellets, phospholipid extraction from pellets used the Bligh and Dyer method, transesterification to FAMEs by treatment with a potassium hydroxide (KOH)/methyl alcohol (MeOH) solution (0.5 mol/L) for 10 min at room temperature, and extraction using hexane (2 mL) FAMEs were analyzed using capillary column gas chromatography (GC).

Results

1 Formulation of Microemulsions

Oil mixtures were prepared using fish oils, Medium chain triglycerides oil and alfa-tocopherol oil. Water Phase was prepared by adding to the water solution all the water-soluble component one by one. Oil phase was added drop-wise to the water phase under agitation.

1.1 Emulsion characterization

The formulation chosen in addition to the active component DHA, in the form of oil containing triglycerides with a high degree of purity and low content of saturated and trans fatty acids, contains other components, which act as carriers and adjuvants to increase bioavailability and optimal protection and incorporation of DHA into cell membranes.

FAME	(µg/mL %)
12:0	0,12
14:0	0,20
16:0	0,58
9c16:1	0,16
18:0	0,29
9c18:1	0,50
11c18:1	0,13
18:2 ω6	0,19
20:4 ω6	0,34
20:5 ω3	7,91
22:5 ω3	2,96
T22:6 ω3	0,2
22:6 ω3	86,13
total SFAs	1,19
Total MUFAs	0,78
PUFAs omega-6	0,53
PUFAs omega-3	97,00

TAB 3: Fatty Acids Methyl Esters (FAMEs) composition of microemulsion.

For reasons of secrecy no further details on the composition can be provided.
The size (μm) of the dispersed lipid particles and the composition as a quantitative percentage (μg/mL %) of the fatty acid content of the emulsion under study were characterized.
The fatty acid composition was evaluated by Gas Chromatographic analysis by transforming the lipid content, essentially consisting of triglycerides, into the corresponding methyl-ester fatty acids by means of a transesterification reaction. Table 1 shows the high content of DHA and the low content of saturated and trans fatty acids.
The dimensions of the particles were evaluated by analysis with DLS (Dinamic Light Scattering) obtaining an average diameter equal to 1.2 ± 0.041 micrometers, therefore the product obtained can be defined as micro-emulsion.

1.2 Physical stability

This emulsion was stored 4 and 20 °C for 90 days to evaluate their physical stability. The dependence of the mean particle size did not depend strongly on storage time but depends on the storage temperature (Table 4).
At low temperatures the emulsion' s particle size increased (Figure 25).
The fact that there was no significant change in the mean particle size of the emulsions after they were held at 20 for 90 days, suggests that they were stable against droplet growth from flocculation, coalescence (Figure 26)**.**

	nm	**nm**
0	1059,15	1059,15
1	1067,85	1069,85
14	1154,78	1400,78
30	1700,1	1400,1
45	1534,1	1379,1
60	1300,1	1300,1
75	1100,1	1350,1
90	1221	1350,1
	20 °C	**4 °C**

TAB 4: Dimension trend comparison between microemulsion stored at 4 °C for 90 days

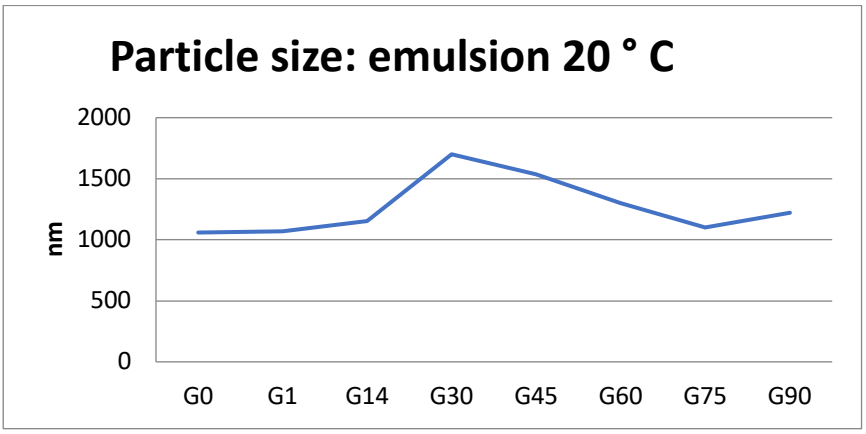


FIG 25: Dimension Trend (nm) of lipid particle of microemulsion stored at 20 °C

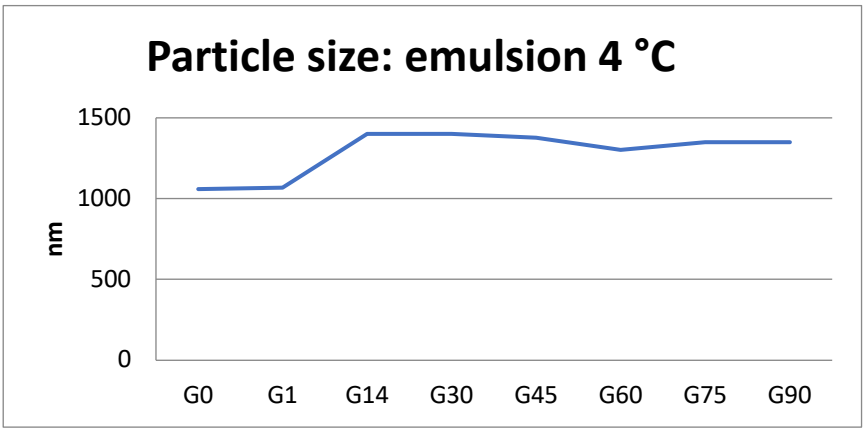


FIG 26: Dimension Trend (nm) of lipid particle of microemulsion stored at 4°C

1.3 Oxidative stability of emulsions

Lipid oxidation in emulsions typically occurs at the oil-water interface due to the interaction of free radicals with unsaturated lipids within the droplets [188] [189]. In these systems, lipid oxidation is usually catalyzed by transition metals, such as iron, which are also responsible for accelerating lipid oxidation by decomposing hydroperoxides into free radicals [189]. The rate of lipid oxidation in emulsions is therefore dependent on the relative location of lipid substrates (polyunsaturated fatty acids) and pro-oxidants (transition metals and hydroperoxides) in the system [189].

In the current study, levels of DHA were measured to monitor the primary oxidation products of the emulsions throughout a 90-day oxidation study.

By adding 3 different antioxidants into the emulsion we were able to protect the lipids peroxidation of DHA (Fig 27).

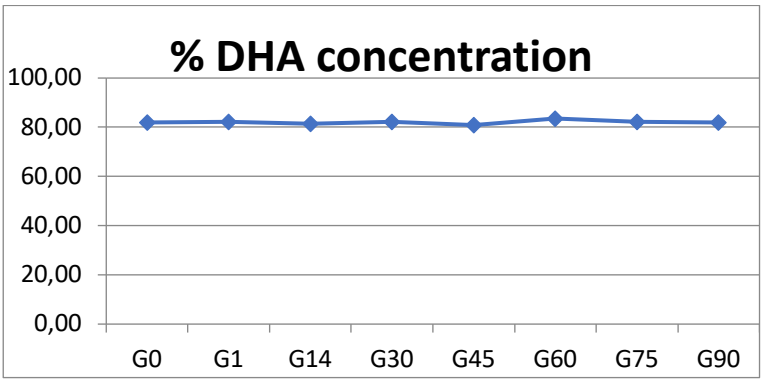


FIG 27: Levels of DHA monitored in 90 days at 20 °C

% µg/mL	G0	G1	G14	G30	G45	G60	G75	G90
SFA	1,02	1,14	1,09	1,15	1,18	1,22	1,25	1,28
MUFA	0,78	0,73	0,80	0,79	0,79	0,80	0,81	0,82
PUFA ω6	0,54	0,54	0,63	0,66	0,71	0,75	0,80	0,84
PUFA ω3	96,60	96,59	96,55	96,53	96,50	96,48	96,45	96,42
Tot TFAs	0,91	0,71	0,70	0,56	0,45	0,34	0,24	0,13

TAB 5: Levels of FAMEs families monitored in microemulsion stored at 4 °C for 90 days

2. Analysis of competitors' s nutraceuticals

We performed the analysis of the triglyceride fractions contained in nutraceutical and food formulas available on the market in Italy, Spain and Japan.

As matter of facts, analytical protocols as quality control of nutraceuticals are important for the interest of producers and consumers, and recently several papers have raised attention on the presence of saturated and trans fatty acids in commercial products of different countries.

Nutraceutical manufacturers are generally concerned with ensuring the absence of heavy metals and other carcinogenic pollutants, such as dioxins and polychlorinated biphenyls, present in the marine habitat. However, the current legislation does not yet take into account other pollutants such as those deriving from industrial processes that induce significant transformations of natural oils.

A total of 9 commercially available ω-3 supplements were analyzed. Among them, products 1-6 were ω-3 supplements present in the Japan market, made of fish oil. Products 7, 8 and 9 were nutraceuticals present in the European market, products 7 and 9 were Italian nutraceuticals, while products 8 was Spanish. Products 7 was made of Krill oil, products 8 was made of fish oils and products 9 was made of algal oil.

Fatty acid methyl esters (FAMEs) are expressed as µg/mL percentages of the total fatty acids in the chromatograms (>98% of the total peak areas of the chromatogram). Results are expressed as quantitative percentages (% µg/mL) ± SD (n=3).

	1	2	3	4	5
14:0	0,44 ± 0,03	0,95 ± 0,03	3,31 ± 0,03	3,20 ± 0,08	7,60 ± 0,13
16:0	3,38 ± 0,08	3,15 ± 0,04	8,83 ± 0,10	9,39 ± 0,19	17,29 ± 0,12
9c16:1	1,30 ± 0,02	1,57 ± 0,03	5,54 ± 0,04	4,21 ± 0,10	9,76 ± 0,07
17:0	0,15 ± 0,04	0,24 ± 0,01	0,41 ± 0,02	0,61 ± 0,02	0,49 ± 0,01
18:0	4,79 ± 0,12	2,25 ± 0,02	3,51 ± 0,03	2,91 ± 0,01	3,31 ± 0,03
9t18:1	0,04 ± 0,01	0,02 ± 0,00	0,08 ± 0,01	0,08 ± 0,00	0,05 ± 0,03
9c18:1	7,83 ± 0,10	8,72 ± 0,15	10,20 ± 0,07	9,12 ± 0,06	12,01 ± 0,07
11c18:1	4,17 ± 0,08	1,77 ± 0,04	2,24 ± 0,06	1,82 ± 0,09	3,15 ± 0,05
t18:2	0,06 ± 0,01	0,06 ± 0,00	0,09 ± 0,01	0,08 ± 0,01	0,23 ± 0,05
18:2 ω6	1,13 ± 0,01	0,94 ± 0,01	3,42 ± 0,02	0,94 ± 0,01	1,79 ± 0,00
18:3 ω3	0,72 ± 0,01	0,51 ± 0,03	10,43 ± 0,11	0,55 ± 0,02	0,91 ± 0,00
20:3 ω6	0,32 ± 0,02	0,18 ± 0,00	0,13 ± 0,01	0,11 ± 0,00	0,14 ± 0,03
20:4 ω6	2,56 ± 0,04	2,13 ± 0,01	2,08 ± 0,03	2,80 ± 0,03	1,54 ± 0,06
t20:5 ω3	0,13 ± 0,02	0,04 ± 0,01	0,72 ± 0,07	0,55 ± 0,02	0,09 ± 0,02
20:5 ω3	54,37 ± 0,49	17,36 ± 0,06	15,21 ± 0,15	12,81 ± 0,02	23,18 ± 0,06
22:5 ω3	4,51 ± 0,03	4,09 ± 0,02	2,14 ± 0,03	2,31 ± 0,03	2,54 ± 0,01
22:6 ω3	13,95 ± 0,04	54,68 ± 0,36	30,96 ± 0,04	47,63 ± 0,26	15,82 ± 0,21
t22:6 ω3	0,18 ± 0,02	1,34 ± 0,08	0,71 ± 0,02	0,89 ± 0,01	0,10 ± 0,01
total SFAs	8,76 ± 0,26	6,60 ± 0,11	16,06 ± 0,17	16,11 ± 0,30	28,69 ± 0,29
TOTAL PUFAs	77,55 ± 0,64	79,88 ± 0,49	64,36 ± 0,40	67,15 ± 0,37	45,93 ± 0,37
SFAs/PUFAs	0,11 0,41	0,08 0,23	0,25 0,43	0,24 0,80	0,62 0,77
TOTAL PUFAs OMEGA-3	73,54 ± 0,57	76,64 ± 0,47	58,73 ± 0,33	63,30 ± 0,33	42,45 ± 0,28
TOTAL TFAs	0,41 ± 0,05	1,46 ± 0,09	1,60 ± 0,10	1,59 ± 0,04	0,47 ± 0,11

	6	7	8	9
14:0	7,34 ± 0,09	13,80 ± 0,95	10,74 ± 0,79	0,01 ± 0,00
16:0	11,39 ± 0,14	20,97 ± 0,94	24,39 ± 1,40	0,04 ± 0,00
9c16:1	6,55 ± 0,13	10,46 ± 0,41	3,30 ± 0,28	0,06 ± 0,00
17:0	0,18 ± 0,02	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
18:0	2,14 ± 0,04	1,16 ± 0,19	0,90 ± 0,04	0,01 ± 0,01
9t18:1	0,12 ± 0,02	0,13 ± 0,02	0,06 ± 0,11	0,00 ± 0,00
9c18:1	6,13 ± 0,08	10,05 ± 0,78	2,59 ± 0,12	0,19 ± 0,01
11c18:1	2,49 ± 0,11	5,33 ± 0,46	4,63 ± 0,15	0,07 ± 0,01
t18:2	0,22 ± 0,03	0,17 ± 0,04	0,00 ± 0,00	0,01 ± 0,00
18:2 ω6	0,72 ± 0,06	1,26 ± 0,03	1,55 ± 0,23	0,03 ± 0,00
18:3 ω3	0,38 ± 0,00	0,78 ± 0,06	0,21 ± 0,03	0,02 ± 0,00
20:3 ω6	0,12 ± 0,01	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
20:4 ω6	2,58 ± 0,03	0,38 ± 0,03	0,30 ± 0,05	0,60 ± 0,12
t20:5 ω3	0,06 ± 0,04	0,27 ± 0,02	0,00 ± 0,00	0,10 ± 0,00
20:5 ω3	37,62 ± 0,24	27,12 ± 1,28	1,89 ± 0,10	3,41 ± 0,02
22:5 ω3	4,58 ± 0,08	0,33 ± 0,05	0,38 ± 0,02	2,18 ± 0,04
22:6 ω3	17,14 ± 0,40	7,79 ± 0,19	49,06 ± 2,66	90,75 ± 0,23
t22:6 ω3	0,23 ± 0,06	0,00 ± 0,00	0,00 ± 0,00	2,53 ± 0,09
total SFAs	21,05 ± 0,29	35,93 ± 2,08	36,02 ± 2,23	0,06 ± 0,01
TOTAL PUFAs	63,14 ± 0,82	37,66 ± 1,64	53,40 ± 3,08	96,98 ± 0,42
SFAs/PUFAs	0,33 0,35	0,95 1,27	0,67 0,72	0,00 0,03
TOTAL PUFA OMEGA-3	59,72 ± 0,71	36,02 ± 1,58	51,55 ± 2,81	96,35 ± 0,30
TOTAL TFAs	0,63 ± 0,16	0,57 ± 0,09	0,06 ± 0,11	2,64 ± 0,09

TAB 6: FAMEs composition of triglycerides in nutraceutical fractions contained in nutraceutical and food formulas available on the market in Italy, Spain and Japan

The GC analyses showed that these products markedly differed one from the other. This was somehow expected since some products declared their lipid content in the labels.

Although all products claimed to contain fish or algal oil, in some of them PUFAs were not the only abundant fatty acid family. It is known that PUFA content is strongly conditioned by the fish species used, the type of food and the environmental conditions of breeding [217] [218]. For example, fish living in cold waters generally have a high ω-3 PUFA content, which guarantees the fish survival in very cold water by maintaining membrane fluidity.

For example, Product 7 was found to contain more than 35% of SFAs (35.93 ±2.08) and less than 40% (36.02±1.58) of ω-3 PUFAs, corresponding to SFA/PUFA ratio of 0.95±1.27.

Only one sample, i.e. Product 9, was constituted almost exclusively (>95%) by ω-3 PUFAs (96.35±0.30). Products of this quality are usually obtained by subjecting the raw materials to the winterization process.

Winterization is a technique of fractional crystallization that requires slow cooling, which allows the separation of based on their melting temperature. In this way, waxes and saturated fats, that are characterized by high melting temperatures, can be solidified and separated by the liquid oily bulk by slowly decreasing the preparation temperature.

Unfortunately, also in this sample TFA derivatives were detected, corresponding to 2.64±0.09 and of the total fatty acids, of which 2.53±0.09 corresponded to trans-DHA. Considering the high quantity of DHA in the capsules (90.75±0.23), the percentage of trans-DHA referred to the total DHA was 2.90%.

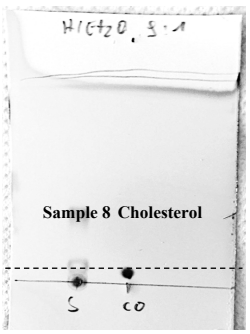
These data provide important information regarding the quality of the starting raw materials used for supplement preparation. However, considering the amount of PUFAs over the total fatty acids, it is not sufficient to establish the quality of the product under investigation. Indeed, focusing on DHA, all the samples analyzed, with the exception of Product 7 and Product 8, contained trans isomers of DHA, in percentages ranging from 0.23±0.06 up to 2.53±0.09 of the total fatty acid content.

On the other hand, Product 7, that do not contain trans-isomers of DHA, contained a few amounts of cis DHA, the lipid which originates trans-isomers.

Apart from DHA mono-trans FAs, other trans-fatty acid derivatives were identified using trans reference libraries synthesized by our group. In particular, mono-trans isomers of oleic (9c, 18:1), arachidonic (20:4 ω-6) acids were individuated. A significant percentage on mono-trans isomers of EPA (20:5 ω-3) was detected, especially in some of those samples that contain more than 15% of EPA, such as product 3 (EPA content of 15.21±0.15) and product 7 (EPA content of 27.12±1.28) that were found to have 0.72±0.07 and 0.27±0.02% of mono-trans isomers of EPA, respectively.

Additionally, since almost all the analyzed samples contained detectable amounts of trans lipids, this research underline the importance of the full trans-isomers characterization of the most common polyunsaturated fatty acids.

Figure 28 shows the lipid composition of sample 8 made of Krill oil. Over the presence of phospholipids, it can be observed also the presence of cholesterol.



Mobile phase :
Hexane: Ether = 9:1

FIG 28: TLC of sample 8; nutraceutical product made of Krill oil.

3. Bioavailability of Olis and Emulsions in Cell Cultures

Recent observations revealed that supplementation of cultured mammalian mast cells (rat basophilic leukemia cells (RBL) with PUFAs Omega-3, in particular docosahexaenoic acid (DHA) leads to robust incorporation of this dietary PUFA into membrane lipids. We observed similar effects in isolated human Ovarian cancer (HELA), Medulloblastoma (DAOY) and Human Embryonic Kidney (HEK), confirming that uptake and incorporation of exogenous DHA into membrane lipids is not cell-type specific.

In each experiment cell lines were treated with the same micromolarity of TG derived from pure fish oil, because this is believed to be the suitable concentration both to promote normal cell proliferation and a response to treatment.

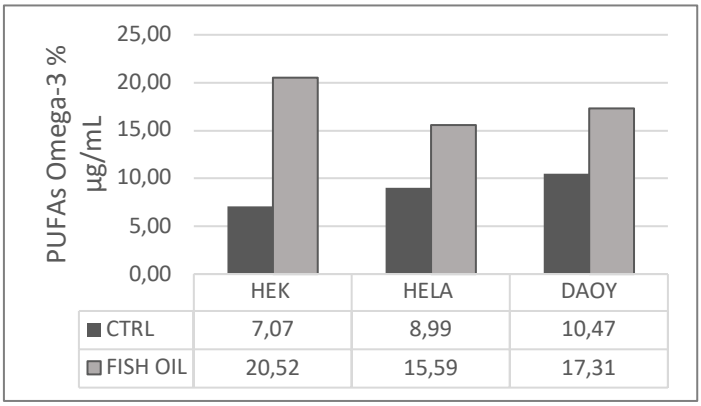


Figure 29: PUFAs Omega-3 incorporation in different cell lines: comparison between control group (CTRL) and cell cultures treated with 1.5 mM of pure fish oil

In order to evaluate the capacity of incorporation of DHA incorporated by the microemulsion in cell membranes, we subjected 3 cell cultures (HEK, DAOY and HELA) to a treatment with 15 µM of the full composition of our microemulsion, 1.5 mM of pure fish oil and 15 µM of an emulsion O/A containing the same concentration of fish oil as present in the microemulsion but without antioxidants and carriers.

DAOY	CTRL		FISH OIL		O/A EMULSION		MICRO EMULSION	
FAME (µg/mL %)	**media**	**dev std**	**media**	**dev std**	**media**	**dev std**	**media**	**dev std**
14:0	0.66	0.05	0.65	0.03	0.66	0.04	0.71	0.04
16:0	19.43	1.44	18.53	0.57	19.54	0.53	17.98	0.68
6c16:1	1.44	0.04	1.38	0.04	1.40	0.02	1.43	0.08
9c16:1	2.60	0.06	2.54	0.06	2.48	0.11	2.57	0.17
18:0	10.99	0.37	10.83	0.23	11.29	0.23	11.29	0.40
9t18:1	0.05	0.06	0.10	0.02	0.09	0.05	0.09	0.13
9c18:1	25.94	0.34	23.45**	0.33	24.70	0.27	24.13*	1.01
11c18:1	5.68	0.06	4.90***	0.07	5.29*	0.23	4.94***	0.07
T 18:2	0.15	0.01	0.15	0.01	0.15	0.03	0.17	0.03
18:2 ω6	5.66	0.15	5.07	0.06	5.55	0.26	5.24	0.40
20:3 ω6	1.13	0.08	1.20	0.03	1.18	0.03	1.34**	0.02
20:4 ω6	15.74	0.59	13.85**	0.29	14.99	0.43	13.87*	0.60
t20:4 ω6	0.06	0.02	0.06	0.01	0.05	0.00	0.07	0.03
20:5 ω3	2.07	0.05	3.29***	0.19	2.43	0.10	2.91***	0.13
22:5 ω3	3.64	0.21	3.70	0.02	3.63	0.05	3.97	0.07
22:6 ω3	4.76	0.26	10.32***	0.81	6.59	0.31	9.29***	1.17
SFAs	31.08	1.84	30.01	0.81	31.49	0.64	29.98	1.11
MUFAs	35.66	0.46	32.26**	0.47	33.87	0.59	33.07**	1.30
PUFAs Omega-6	22.53	0.82	20.12*	0.31	21.71	0.62	20.45*	0.96
PUFA Omega-3	10.47	0.52	17.31***	0.99	12.64	0.43	16.17***	1.35
SFAs/MUFAs	0.87	0.06	0.93	0.03	0.93	0.03	0.91	0.07
Omega 6/Omega 3	2.15	0.03	1.17***	0.09	1.72**	0.11	1.27***	0.17
TFAs	0.26	0.05	0.30	0.03	0.29	0.07	0.33	0.19

TAB 7: Membrane remodeling in DAOY cells after 24h treatment with 1.5 mM of FISH OIL, 15 µM of O/A EMUSION and 15 µM of MICRO EMULSION.

HELA	CTRL		FISH OIL		O/A EMULSION		MICRO EMULSION	
	media	**devstd**	**media**	**devstd**	**media**	**devstd**	**media**	**devstd**
14:0	0,78	0,00	0,81	0,05	0,77	0,04	0,86	0,06
16:0	20,82	0,57	18,46*	0,54	18,97*	0,48	18,58*	0,24
6c16:1	2,92	0,17	2,60	0,05	3,01	0,23	2,54	0,10
9c16:1	2,06	0,01	1,96	0,08	1,93	0,06	1,94	0,06
18:0	13,74	0,22	13,48	0,13	13,64	0,21	12,75*	0,26
9t18:1	0,31	0,10	0,11	0,02	0,16	0,05	0,11	0,03
9c18:1	25,34	0,19	23,35*	0,06	24,15	0,47	23,18**	0,99
11c18:1	4,51	0,02	4,08	0,09	4,35	0,11	4,10	0,28
T 18:2	0,18	0,06	0,08	0,02	0,11	0,04	0,08	0,04
18:2 ω6	4,58	0,11	4,36	0,17	4,78	0,13	4,18	0,19
20:3 ω6	1,07	0,04	1,16	0,06	1,24	0,09	1,09	0,05
20:4 ω6	14,58	0,67	13,91	0,47	15,20	0,56	12,46*	0,73
t20:4 ω6	0,14	0,02	0,04	0,01	0,07	0,05	0,09	0,02
20:5 ω3	2,70	0,11	4,39***	0,11	3,43**	0,06	4,02***	0,29
22:5 ω3	2,50	0,01	2,48	0,02	2,39	0,05	2,93**	0,17
22:6 ω3	3,78	0,08	8,72**	0,57	5,80	0,82	11,09***	2,19
SFAs	35,34	0,35	32,75***	0,46	33,38**	0,24	32,19***	0,56
MUFAs	34,82	0,36	32,00*	0,25	33,43	0,42	31,76**	1,41
PUFAs Omega-6	20,22	0,82	19,43	0,69	21,22	0,75	17,73*	0,94
PUFA Omega-3	8,99	0,07	15,59**	0,47	11,62	0,81	18,04***	2,64
SFAs/MUFAs	1,01	0,00	1,02	0,02	1,00	0,02	1,01	0,04
Omega 6/Omega 3	2,25	0,08	1,25***	0,08	1,83**	0,20	1,00***	0,19
TFAs	0,62	0,18	0,22*	0,03	0,34	0,12	0,28*	0,09

TAB 8: Membrane remodeling in HELA cells after 24h treatment with 1.5 mM of FISH OIL, 15 μM of O/A EMUSION and 15 μM of MICRO EMULSION

TAB 9: Membrane remodeling in HEK cells after 24h treatment with 1.5 mM of FISH OIL, 15 µM of O/A EMUSION and 15 µM of MICRO EMULSION.

HEK	CTRL		FISH OIL		O/A EMULSION		MICRO EMULSION	
FAME (µg/mL %)	**media**	**dev std**	**media**	**dev std**	**media**	**dev std**	**media**	**dev std**
14:0	1,45	0,30	1,04	0,08	1,12	0,10	1,23	0,16
16:0	26,20	0,84	21,55**	1,59	24,97	0,14	25,69	0,52
6c16:1	3,35	0,76	2,45**	0,23	2,72	0,07	3,10	0,58
9c16:1	2,90	0,49	1,89	0,18	2,12	0,15	2,41	0,19
18:0	13,45	0,48	10,98**	0,26	12,56	0,28	12,39	0,75
9t18:1	0,07	0,05	0,03	0,01	0,03	0,01	0,04	0,03
9c18:1	23,41	1,27	20,59**	0,23	22,46	0,59	21,61	0,63
11c18:1	4,53	0,52	4,24	0,06	4,91	0,08	4,31	0,24
T 18:2	0,11	0,03	0,04	0,01	0,06	0,01	0,07	0,01
18:2 ω6	4,82	0,65	4,08	0,46	4,88	0,15	5,33	0,55
20:3 ω6	0,98	0,46	0,30	0,11	0,32	0,10	0,54	0,06
20:4 ω6	11,45	1,86	12,23	0,47	15,12*	0,87	12,97	1,23
t20:4 ω6	0,20	0,08	0,05	0,02	0,06	0,02	0,11	0,02
20:5 ω3	0,54	0,09	2,57***	0,43	1,20	0,09	1,28*	0,13
22:5 ω3	1,09	0,24	1,94**	0,13	1,71*	0,14	1,48	0,15
22:6 ω3	5,44	0,59	16,01***	2,95	5,76	0,61	7,45*	0,62
SFAs	41,10	1,23	33,57***	1,92	38,66	0,32	39,31	1,11
MUFAs	34,19	0,96	29,16***	0,53	32,20	0,63	31,42	1,15
PUFAs Omega-6	17,25	1,38	16,62	1,02	20,32*	0,91	18,84	0,68
PUFA Omega-3	7,07	0,57	20,52***	3,40	8,68	0,55	10,21	0,55
SFAs/MUFAs	1,20	0,06	1,15	0,05	1,20	0,03	1,25	0,08
Omega 6/Omega 3	2,45	0,34	0,83***	0,21	2,35	0,27	1,85	0,16
TFAs	0,38	0,16	0,12	0,03	0,15	0,02	0,22	0,03

Supplementation of culture media with PUFAs for 24 hours leads to an important increase in ω−3 PUFA-containing membrane lipids in cells supplemented with DHA. Each treatments results in significantly increased overall unsaturation of membrane lipids.

Tables 7-8 and 9 show membrane fatty acid composition in the cell lines after incubations with O/A emulsion, pure fish oil and micro-emulsion for 24 h. The main fatty acid change detected in the DAOY cell membrane phospholipids after 24 h exposure to different treatments was the increase in each samples of the concentration of DHA.

Incubation with fish oil, O/A emulsion o microemulsion did not cause any significant change in levels of monounsaturated cis-fatty acids; 6c-16:1, 9c-16:1 and 11c-18:1 (vaccenic) in DAOY and HELA cells, while there are significant changes (p=0,01) in HEK cell line.

In DAOY cell line there are significant changes in the samples treated with pure fish oil and with micro-emulsion, but no statistically relative variation in those treated with O/A emulsion.

As mentioned above, in all the samples the phospholipid composition of the membrane was enriched with the omega-3 component. A significant increase ($P < 0.001$) of omega-3 PUFAs is evident both in cells treated with pure fish oil and in those treated with microemulsion. Furthermore, there is a decrease in Arachidonic Acid in both treatments and consequently a significant reduction in the omega6/omega3 ratio.

There was also a significant decline in MUFA shares in both treatments.

In the HELA cell line, statistically significant changes also occur in the group of cells treated with O/A emulsion.

In all the samples the phospholipid composition of the membrane was enriched with the omega-3 component (also DPA), while only in the group treated with micro emulsion there was a significant decrease in the track omega-6, in particular of Arachidonic Acid. Consequently, the differences in the omega6 / omega3 ratio appear statistically significant in all treated groups.

There is also a significant decrease in the shares of SFA in all treatments and MUFA in treatments with pure oil and micro emulsion.

There is also a significant difference in the TFAs between the control group and the treatments with pure fish oil and with micro emulsion.

HELA cells showed a better response to treatment in comparison with DAOY cell line, with the same concentration administered there is a more significant increase in the percentage of incorporated Omega - 3 Fatty Acids.

This can be due to:

Lower percentage of omega-3 fatty acids in the control group of HELA cells, compared to the control group of DAOY cells (the omega 3 PUFAs are in fact 8.99 and 10.47 respectively), which results in a higher demand for PUFA in the membrane, therefore easier incorporation.

Lower percentage of total MUFAs in the HELA cell control group compared to the DAOY cell control group (34.8 and 35.7 respectively).

While in the DAOY and HELA cell lines no significant differences are observed between the amount of DHA incorporated in the cells treated with pure fish oil and those treated with microemulsion, in the HEK cell line there is a significant difference between the amount of DHA incorporated in the cells treated with pure fish oil and those treated with micro emulsion and O/A emulsion, suggesting that further investigations are needed.

Despite the copious incorporation of exogenous PUFAs into membrane lipids and the resulting increase in overall membrane unsaturation, cells in vitro did not show any differences in proliferation at these levels of supplementation.

Thus, we hypothesized that mammalian cells may compensate for perturbations from exogenous FAs by remodeling their lipidomes. Indeed, DHA supplementation reduced the abundance of other polyunsaturated lipid species This effect could potentially be explained by replacement of these PUFA-containing lipids by DHA-containing ones.

4. First test of human supplementation

To confirm that the membrane incorporation of supplemented PUFAs in cultured cells appropriately recapitulates in vivo conditions, we analyzed membrane lipidomes in 5 healty adults volunteers.
The volunteers were currently undergoing a dose (5 mL containing 261 mg of DHA) of micro emulsion taken daily in the evening during dinner for 2 months.
At the end of each month of supplementation, the blood samples of the volunteers were analyzed in order to evaluate the concentration of DHA incorporated in the cell membranes of mature erythrocytes.
The t0 corresponds to the result of the analysis before the start of the supplementation.
t1 = 1 month of supplementation;
t2 = 2 months of supplementation.

% µg/mL	t0	t1	t2
DHA	4,4±0,9	5,3±1,8	7,6±1,6
PUFAs OMEGA-3	5,2±0,9	7,2±2,6	7,8±2,1

TAB 10: DHA and PUFAs Omega-3 incorporation in 5 healthy adult volunteers before the supplementation with Micro Emulsion (t0), after one month (t1) and after two months (t2)

The incorporation of DHA from the micro emulsion supplementation into membrane lipids in healty adult volunteers led to remarkably similar lipidomic remodeling as in cultured cells.

Conclusions

The analysis of the lipidomic profile of the mature erythrocyte membrane is a driving element for the creation of new nutraceutical lines based on the nutrilipidomic approach: the individuation of the unbalances of the membrane fatty acid composition in subjects having specific health conditions, compared to healthy population, can be used to choose the right active ingredients, and to create new microemulsion formulations addressed to the re-equilibrium of the membrane compartment, using the natural remodeling process used by our cells.

In the industrial PhD work the main aim was to develope a DHA-containing nutraceutical formulation in the form of emulsion, overcoming the difficulty of the capsule ingestion, to be administered orally, targeting the fragile and infant populations. Each ingredient of the formulation has been studied on the basis of the data collected on membrane lipidomic profiles in health conditions where DHA deficit is observed, in particular taking into account the lipidome data available on the autistic children population.

The micro-emulsions formulated was tested to guarantee the physical and the chemical stability of the components, monitoring parameters such as particle size and degree of oxidation of the active ingredient DHA.

The optimal incorporation of DHA as active ingredient present in the microemulsion was evaluated using in vitro experiments in appropriate cell culture models, also to understand the differences with the pure oil and the simple O/W emulsion administration, and was then preliminarily tested in humans, administering it to a small group of volunteers, in order to assess the effectiveness of DHA incorporation from the new formulation, using the erythrocyte membrane lipidome analysis.

This work opens the way to a new nutraceutical line of the company Lipinutragen, where the thesis was developed as part of the R&D projects. The line has been registered with the trademark of Nutraomic®, and in January 2020 the new product Li DHA was launched to the market (www.lidha.it).

On the other hand, it is worth underlining that the PhD work included a thorough experience of the PhD candidate in the field of lipidomics, experiencing the study of lipidomic profiles in obesity, as well as plant lipidomics, experiencing the metabolic changes that occur in seed germination.

Moreover, in the field of pharmaceutical and nutraceutical formulations, the PhD candidate experienced the subject of liposome encapsulation, in particular with the result of prolonging the life time in human plasma of the peptide somatostatin. In all these topics, the PhD candidate

participated to the activities of the CNR group of her supervisor, gaining a multidisciplinary competence and, last but not least, publishing the results in international scientific journal with good impact factor.

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