INSIGHT INTO THE CONTRIBUTION OF ENDOGENOUS CAROTENOIDS OF PHOTOAUTOTROPHIC MICROALGAE TO THE OXIDATIVE STABILITY OF N-3 LC-PUFA

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De route

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>
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Robbe



SAMENVATTING

Zowel vis als visolie zijn rijke bronnen van langketen omega-3 poly-onverzadigde vetzuren (n-3 LC-PUFA). Als ze deel uitmaken van de voeding, dragen ze in belangrijke mate bij tot de aan n-3 LC-PUFA gerelateerde gezondheidsvoordelen waaronder de preventie van hart- en vaatziekten [1]. Duurzaamheidsproblemen als gevolg van een gelimiteerde visvoorraad maken het echter onmogelijk om enkel op vis te berusten als n-3 LC-PUFA bron. Dit geldt zowel op een directe als indirecte manier (via aquacultuur) [2,3]. Door een toenemende vraag naar n-3 LC-PUFA bronnen als gevolg van een stijgend bevolkingsaantal en een hoger gezondheidsbewustzijn, is dit probleem alleen maar groter geworden. Hierdoor is ook het onderzoek naar alternatieve n-3 LC-PUFA bronnen toegenomen [4]. Microalgen, de eencellige, primaire n-3 LC-PUFA producenten aan de basis van de aquatische voedselketen, toonden al potentieel. De kweek ervan kan een nieuwe n-3 LC-PUFA voorraad genereren in plaats van het uitputten van een bestaande, en hun toevoeging aan voedingsmiddelen (voedselverrijking) zou de gezondheidsbewuste consument een veganistische mogelijkheid bieden om de inname van voldoende n-3 LC-PUFA te garanderen [5,6]. De wereldwijde visvoorraad kan deze mogelijkheid niet bieden. Bovendien is bekend dat vis(olie) erg gevoelig is aan chemisch bederf onder de vorm van lipidenoxidatie. Vooral n-3 LC-PUFA zijn hier omwille van hun structuur namelijk erg gevoelig voor [7]. Uit voorgaand onderzoek is echter bekend dat bepaalde microalgen, en de voedingsmiddelen waarmee ze verrijkt zijn, oxidatief stabiele producten kunnen opleveren [8]. Hoewel dit werd toegewezen aan de aanwezigheid van endogene carotenoïden, ontbrak verder inzicht in hun antioxidatieve rol [9]. Het hoofddoel van dit doctoraatsonderzoek was daarom om deze oxidatieve stabiliteit van microalgen verder te onderzoeken en tegelijkertijd de antioxidatieve capaciteit van hun carotenoïden te ontrafelen. Hoewel het onderzoek een eerder fundamentele dan praktische insteek had, zou inzicht hierin belangrijke aandachtspunten kunnen onthullen voor de ontwikkeling van n-3 LC-PUFA rijke voedingsmiddelen met behulp van microalgen of hun componenten.

Het experimentele werk bestond uit het onderzoeken van deze antioxidatieve rol van endogene carotenoïden in zowel waterige biomassasuspensies (Hoofdstukken 3 en 4) als bulkoliesystemen (Hoofdstukken 5 en 6).

Voor de waterige suspensies, werd in Hoofdstuk 3 de oxidatieve stabiliteit van twee veelbelovende microalgen met een duidelijk verschil in carotenoïdenprofiel

onderzocht (*Nannochloropsis* en *Phaeodactylum*). De resultaten toonden een duidelijk verschil in oxidatieve stabiliteit: *Nannochloropsis* leidde tot oxidatief onstabiele en *Phaeodactylum* tot oxidatief stabiele producten. Dit wees op een effect van het carotenoïdengehalte en (mogelijks) -type. Hieruit blijkt *Phaeodactylum* dan ook geschikter te zijn voor voedselverrijking. In Hoofdstuk 4 werd vervolgens onderzocht of dit verrijkingspotentieel ook geldt onder belichte bewaarcondities. Dit aangezien chlorofyl, een pigment dat overvloedig aanwezig is in fotoautotrofe microalgen, bekend staat om zijn oxidatie bevorderende, fotosensibiliserende eigenschappen. Het optreden van een geringe hoeveelheid foto-oxidatie leidde echter niet tot oxidatieve instabiliteit.

Voor de bulkoliesystemen werd *Phaeodactylum* in Hoofdstuk 5 als alternatieve bron van natuurlijke antioxidanten beschouwd bovenop zijn potentieel als n-3 LC-PUFA alternatief. Het mengen van visolie als oxidatief onstabiele n-3 LC-PUFA bron met *Phaeodactylum* biomassa remde de oxidatie niet, ondanks een detecteerbare carotenoïdenmigratie van biomassa naar olie. Een vervolgexperiment, waarbij verschillende verhoudingen visolie en *Phaeodactylum* totaal lipidenextract gemengd werden, toonde aan dat vooral de hoeveelheid carotenoïden (voornamelijk fucoxanthine in het geval van *Phaeodactylum*) in verhouding tot de hoeveelheid n-3 LC-PUFA bepalend is voor de oxidatieve stabiliteit. De veralgemeenbaarheid van deze bevindingen werd aangetoond in Hoofdstuk 6, waar aan het proefopzet ook totale lipidenextracten van *Tisochrysis* werden toegevoegd. Een beperkte invloed van lipidenklasse samenstelling en het belang van een voldoende hoge initiële hoeveelheid aan carotenoïden werden geïllustreerd.

De resultaten van het huidige doctoraatsonderzoek leidden tot verschillende conclusies van zowel fundamentele als praktische aard. Hoofdstukken 3 en 6 mondden gezien hun meer fundamentele focus voornamelijk uit tot conclusies van de eerste soort. Door een carotenoïdenafbraak doorheen bewaring toonden deze duidelijk het belang van endogene carotenoïden aan voor de stabiliteit van n-3 LC-PUFA. Dit gold voor alle carotenoïden, en zowel in biomassasuspensies als in bulkoliën. Bovendien werd een duidelijk verband met lipidenoxidatie vastgesteld. Ook werd het belang van de (initiële) hoeveelheid bovenop de aanwezigheid aangetoond. Hoewel eenduidige conclusies over de invloed van het type carotenoïde moeilijker te maken waren, wees een vergelijking van carotenoïden binnen een bepaald systeem op de aanwezigheid van deze invloed. Door carotenoïden ook over verschillende systemen heen te vergelijken, werd ook een systeemimpact aangetoond. Conclusies met betrekking tot praktische

toepassingen konden vooral uit Hoofdstukken 4 en 5 worden getrokken. Hoofdstuk 4 toonde aan dat belichting tijdens bewaring van weinig belang is voor de oxidatieve stabiliteit van met microalgen verrijkte levensmiddelen die in transparante verpakkingen worden bewaard. Uit Hoofdstuk 5 bleek dat de toevoeging van *Phaeodactylum* biomassa geen antioxidatief potentieel heeft om n-3 LC-PUFA rijke visolie te stabiliseren. De endogene carotenoïden hebben dat wel, hoewel daarvoor hoge (initiële) hoeveelheden nodig zijn.

Finaal kon gesteld worden dat de ene fotoautotrofe microalg de andere niet is. Bij het realiseren van hun voordeel om tot oxidatief stabiele n-3 LC-PUFA rijke voedingsmiddelen te leiden, is de hoeveelheid carotenoïden van cruciaal belang. Voor het toepassen van n-3 LC-PUFA rijke microalgen in de voedingsproducten van de toekomst, moeten echter nog verschillende drempels overwonnen worden met betrekking tot de hoge productiekosten, de wetgeving en de organoleptische aanvaardbaarheid voor de consument. Desalniettemin blijft het potentieel van microalgen om bij te dragen aan de duurzaamheids- en stabiliteitsproblemen van n-3 LC-PUFA rijke producten duidelijk.

ABSTRACT

Both fish and fish oil are rich long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFA) sources, that are, if part of the diet, important contributors to the n-3 LC-PUFA related health benefits. Amongst others, these include the prevention of cardiovascular diseases [1]. Sustainability issues resulting from the finite character of the fish stock however pose a threat on solely relying on fish, both directly and indirectly (via aquaculture), as traditional n-3 LC-PUFA source [2,3]. As the demand for n-3 LC-PUFA sources is growing due to a rising population and an increased health benefit awareness, this problem only became more prominent and has driven the search for novel n-3 LC-PUFA sources [4]. Microalgae, the unicellular, primary producers of n-3 LC-PUFA at the base of the aquatic food chain, have already shown their potential. Cultivating them would generate a new supply of n-3 LC-PUFA rather than depleting an existing one, and adding them to food products (food enrichment) would offer vegan opportunities to the health-conscious consumer to guarantee the sufficient n-3 LC-PUFA intake for which the global fish stock cannot account [5,6]. Moreover, whereas fish (oil) is known to be prone to the chemical deterioration process of lipid oxidation (n-3 LC-PUFA in particular are very susceptible due to their structure [7]), former research has shown that certain microalgae and with them enriched food products led to oxidatively stable products [8]. This was explained by the presence of antioxidative, endogenous carotenoids, but further insight into their antioxidative role was missing [9]. The main aim of this PhD research was therefore to further investigate this microalgal oxidative stability while unraveling the antioxidative capacity of their endogenous carotenoids. Although the PhD research was more fundamentally than practically oriented, understanding this could reveal important points of interest for developing n-3 LC-PUFA rich food products with microalgae or microalgal compounds.

The experimental work consisted of investigating the endogenous carotenoids' antioxidative role in both aqueous biomass suspensions (Chapters 3 and 4) and bulk oil systems (Chapter 5 and 6).

Regarding the aqueous suspensions, Chapter 3 compared the oxidative stability of two promising microalgae with a clearly different carotenoid profile (*Nannochloropsis* and *Phaeodactylum*). The results showed a clear difference in oxidative stability (*Nannochloropsis* led to oxidatively unstable and *Phaeodactylum* to oxidatively stable products) which pointed out an impact of carotenoid content and (possibly) type. It showed *Phaeodactylum* to be more suitable for food enrichment. Chapter 4 investigated whether this enrichment potential is also valid under illuminated conditions, given that chlorophyll

(abundantly present in photoautotrophic microalgae) is known for its oxidation promoting photosensitizing properties. The appearance of a minor amount of photo-oxidation did however not cause oxidative instability.

Regarding the bulk oil systems, Chapter 5 approached *Phaeodactylum* as an alternative source of novel antioxidants on top of its n-3 LC-PUFA enrichment potential that was shown in Chapters 3 and 4. Mixing fish oil as an oxidatively unstable n-3 LC-PUFA source with *Phaeodactylum* biomass did not inhibit oxidation despite a detectable carotenoid transfer. A follow-up experiment, in which different proportions of fish oil and *Phaeodactylum* total lipid extract were mixed, showed that mainly the amount of carotenoids (predominantly fucoxanthin in the case of *Phaeodactylum*) relative to the n-3 LC-PUFA content determines oxidative stability. The generalizability of these findings was shown in Chapter 6, in which also *Tisochrysis* total lipid extracts were added to the experimental set-up. A minor impact of lipid class composition and the importance of a sufficiently high initial carotenoid amount were illustrated.

The results of the current PhD research led to multiple conclusions with either a fundamental or a practical nature. Given their more fundamental focus, Chapters 3 and 6 mainly led to conclusions of the first kind. By a degradation throughout storage, they clearly showed the importance of endogenous carotenoids for obtaining n-3 LC-PUFA stability, and this for all carotenoids, in both biomass suspensions and bulk oils. A clear link with lipid oxidation was observed, and the impact of (initial) quantity on top of their presence was revealed. Although unequivocal conclusions on the impact of carotenoid type were harder to make, a comparison of carotenoids within a certain system did indicate an impact. By also comparing carotenoids across different systems, the appearance of a system impact was illustrated as well. Conclusions towards practical applications could mainly be drawn from Chapters 4 and 5. Chapter 4 showed illumination throughout storage to be of minor concern when thinking of the enrichment of food products stored in transparent containers. Chapter 5 showed *Phaeodactylum* biomass addition to have no antioxidative potential to stabilize n-3 LC-PUFA rich fish oil. Microalgal carotenoids themselves do possess this potential, but high (initial) amounts are required.

Finally, it could be stated that one photoautotrophic microalga is not the other. In realizing their advantage of giving rise to oxidatively stable n-3 LC-PUFA rich food products, the amount of carotenoids is crucial. For applying n-3 LC-PUFA rich microalgae in future food products however, several challenges still need to be addressed regarding production costs, legislation and consumer acceptance. Nevertheless, their potential in overcoming both the sustainability and stability issues n-3 LC-PUFA rich products face, remains clear.

LIST OF ABBREVIATIONS

AA	Arachidonic acid
ALA	α-Linolenic acid
ANOVA	Analysis of variance
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
C12:0	Lauric acid
C16:0	Palmitic acid
CI	Confirming ion
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
EPA	Eicosapentaenoic acid
FFA	Free fatty acid
FID	Flame ionization detection
FOX	Ferrous oxidation xylenol orange
GC	Gas chromatography
GL	Glycolipid
GMOs	Genetically modified organisms
HPLC	High-performance liquid chromatography
HS-SPME Arrow GC-MS	Headspace Arrow solid phase microextraction gas chromatography coupled with mass spectrometry
LA	Linoleic acid

MS	Mass spectrometry
n-3 PUFA	Omega-3 polyunsaturated fatty acids
n-3 LC-PUFA	Long-chain omega-3 polyunsaturated fatty acids
n-3 SC-PUFA	Short-chain omega-3 polyunsaturated fatty acids
n-6 PUFA	Omega-6 polyunsaturated fatty acids
NL	Neutral lipid
O/W emulsions	Oil-in-water emulsions
PDA	Photodiode array
PL	Phospholipid
PV	Peroxide value
QI	Quantifying ion
ROS	Reactive oxygen species
SDA	Stearidonic acid
SIC	Single ion chromatogram
SPE	Solid phase extraction
SPME	Solid phase microextraction
TAG	Triacylglycerol
TIC	Total ion chromatogram
TPP	Triphenylphosphine
VOC	Volatile organic compound

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CONTEXT AND AIM

Both fish and fish oil are important sources of long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFA). These semi-essential fatty acids have been associated with numerous health benefits, of which especially the ones related to the prevention of cardiovascular diseases (CVD) have been strongly recognized [1]. Nonetheless, their intake is generally (in approximately 80% of the global population) insufficient to perceive these health benefits [10], implying that an increased consumption would be beneficial for human health. The global wild fish stock however struggles with sustainability issues: approximately one third suffers from overfishing (i.e. harvesting more than can be replenished naturally) and only seven percent offers opportunities for growth [3]. Also farmed fish (aquaculture) is usually dependent on wild fish (in the form of fish oil in feeds) for its n-3 LC-PUFA input [2,4]. Therefore, with a demand for n-3 LC-PUFA that is increasing due to both a rising population and an increased health benefit awareness, other n-3 LC-PUFA sources need to be addressed.

Microalgae are a novel n-3 LC-PUFA source with potential. This diverse group of unicellular, aquatic organisms covers the primary production of n-3 LC-PUFA at the base of the aquatic food chain. In this way, they are responsible for most of the n-3 LC-PUFA in aquatic organisms of higher trophic levels [11]. Rather than overexploiting these (mainly fish), cultivating microalgal species that are rich in n-3 LC-PUFA would generate the new supply of n-3 LC-PUFA that is sought for and to which a lot of research has already been conducted in the past years [5,6,12–14]. Implementing the microalgae in the human food chain, by direct consumption or by (either direct or indirect) food enrichment, would offer opportunities for the health-conscious consumer to guarantee a sufficient n-3 LC-PUFA intake in a sustainable way. Moreover, several photoautotrophic microalgal species have been shown to possess the supplemental advantage of being characterized by a high oxidative stability [8].

When considering n-3 LC-PUFA, or food products rich in them, lipid oxidation indeed forms a major point of attention. This chemical deterioration process affects n-3 LC-PUFA in particular due to their chemical structure with multiple unsaturations (double bonds) and bis-allylic hydrogen atoms. The process is undesired as it goes along with the formation of both rancid flavors and potentially toxic compounds and with the loss of nutritional value, since n-3 LC-PUFA can no longer perform their health-beneficial effects upon

degradation [7]. Lipid oxidation can be prevented by the addition of (synthetic or natural) antioxidants. In industry, this is a general practice to prolong the shelf life of food products, especially those prone to lipid oxidation such as n-3 LC-PUFA rich products [15,16]. However, endogenous antioxidants can sometimes suffice to prevent oxidation, as has been shown before for certain photoautotrophic microalgae [8].

In the light of using microalgae as an alternative n-3 LC-PUFA source with a major focus on oxidative stability, the PhD research of Lore Gheysen formed an important preceding research [9]. Targeting on developing n-3 LC-PUFA rich microalgae enriched vegetable-based products, she found that among the endogenous antioxidants especially the presence of carotenoids is important for microalgal n-3 LC-PUFA stability. She observed that photoautotrophic microalgae, which contain endogenous carotenoids, give rise to oxidatively stable products, whereas heterotrophic ones, not containing them, do not. This finding forms the starting point of the current PhD research of which the **general aim** is to further unravel the antioxidative role of microalgal endogenous carotenoids and in that way build further on the insights of microalgal n-3 LC-PUFA stability.

Figure 1 gives an outline of this PhD thesis. The first part (**Part I**) consists of a literature review in two chapters. **Chapter 1** covers a thorough discussion on n-3 LC-PUFA sources, together with their associated health benefits and the importance of a sufficient intake. Both traditional (fish and fish oil) and novel sources are reviewed in terms of 'importance', 'production', 'sustainability' and 'quality'. Next to microalgae, also alternative marine resources and transgenic plants are considered as novel sources allowing an updated overview of the role n-3 LC-PUFA rich microalgae could play in the future n-3 LC-PUFA supply. Also the implementation into the human food chain is discussed. **Chapter 2** deals with the complex problem of lipid oxidation. It gives a general overview of both oxidation and antioxidation in terms of 'definition', 'mechanism', 'affecting factors' and 'analysis', providing the most important knowledge that is necessary to evaluate the results of the current PhD research in a correct way. The chapter ends by narrowing down to the oxidative stability of different n-3 LC-PUFA rich systems. The current knowledge on stability differences and causative factors is discussed, with a focus on photoautotrophic microalgae and fish oil.

Part II comprises the experimental work in four different chapters. The first two (Chapters 3 and 4) study aqueous biomass suspensions as used in the PhD of Lore Gheysen. Model systems rather than real food matrices were considered in this

to enable the focus on microalgal compounds. The subsequent two (Chapters 5 and 6) on the other hand, examine the lipids themselves and therefore consider 'bulk oils' as research matrices. All four experimental chapters pay special attention to the endogenous carotenoids, having in common the aim of getting insight into their antioxidative role. Their evolution throughout storage is studied in most chapters (Chapters 3, 4 and 6). Whereas the focus of Chapters 3 and 6 is purely fundamental, Chapters 4 and 5 also possess a practical aspect (by respectively investigating the potential of enrichment in transparent packages and the potential of using *Phaeodactylum* biomass as a natural antioxidant ingredient as such). In what follows the sequence of the different chapters is further explained.

Chapter 3 starts off with a comparison between the aqueous suspensions of two different photoautotrophic microalgae with a clearly different carotenoid profile (*Nannochloropsis* and *Phaeodactylum*) that formerly showed n-3 LC-PUFA enrichment potential. This carotenoid profile (i.e. composition and content) was analyzed throughout a storage experiment in parallel with the analysis of both n-3 LC-PUFA content and primary and secondary lipid oxidation products to assess oxidative stability. For the analysis of secondary oxidation products, this came along with the implementation of a suitable methodology using solid phase microextraction with Arrow technology as a sampling technique. The resulting 'headspace Arrow solid phase microextraction gas chromatography coupled with mass spectrometry' (HS-SPME Arrow GC-MS) method was used throughout the whole PhD research. It was hypothesized that different carotenoids could degrade differently throughout storage, indicating on their antioxidative role.

Former research did not yet consider photo-oxidative stability when exploring food enrichment with photoautotrophic microalgae. The abundance of chlorophyll, as a well-known photosensitizer in microalgae, could however promote lipid oxidation under the influence of light (i.e. photo-oxidation) and in this way jeopardize the enrichment potential of autoxidatively stable microalgae when food products in transparent containers are envisioned. Therefore, different from the other chapters where only autoxidation (i.e. without illumination) is considered, **Chapter 4** investigates both photo- and autoxidation during storage. *Phaeodactylum* suspensions were selected as autoxidatively stable systems of which it was hypothesized that illumination would promote lipid oxidation due to the presence of chlorophyll as a photosensitizer. *Schizochytrium* suspensions, autoxidatively instable systems lacking both chlorophyll and carotenoids, were also considered. As an instable reference they could learn

something about the extent to which oxidation was promoted. Despite of the photo-oxidation focus, carotenoids were still of particular interest given their reported (photo-oxidation limiting) quenching properties. Their profile throughout storage was again analyzed.

Observing the oxidative stability of *Phaeodactylum* suspensions in both dark (Chapter 3) and illuminated (Chapter 4) storage conditions, raised the question whether this microalga could also be used as novel antioxidant on top of being used as a novel n-3 LC-PUFA source. **Chapter 5** aims to answer this question while also gaining supplemental insight into the antioxidative capacity of the carotenoids (especially fucoxanthin for *Phaeodactylum*) to which the oxidative stability was attributed. In a first set-up, the oxidative stability of fish oil with the addition of 2.5% (w/w) *Phaeodactylum* biomass, for which an oxidation inhibition effect was hypothesized, was investigated. However, detecting no considerable effect in spite of a detectable migration of carotenoids to the fish oil phase, gave rise to a second set-up. For this, fish oil was mixed in different proportions with a *Phaeodactylum* total lipid extract and oxidative stability was again evaluated, showing the amount of carotenoids relative to the amount of n-3 LC-PUFA content to be a good indicator for oxidative stability.

In **Chapter 6**, the generalizability of the findings of Chapter 5 were checked by also studying the extracts of another fucoxanthin and n-3 LC-PUFA rich microalga (*Tisochrysis*) albeit with a different lipid class composition. In this way, also the influence of the latter, of which it was hypothesized that a lower amount of polar lipids is associated with a lower oxidative stability, could be investigated. Furthermore, just like for Chapter 3 and 4, the carotenoid profile was analyzed throughout storage with the aim of getting insight into their antioxidative role albeit in a different system.

Finally, the most important findings of the different experimental chapters were brought together in a concluding **Chapter 7** (**General conclusions**) in which also the future research perspectives are presented.

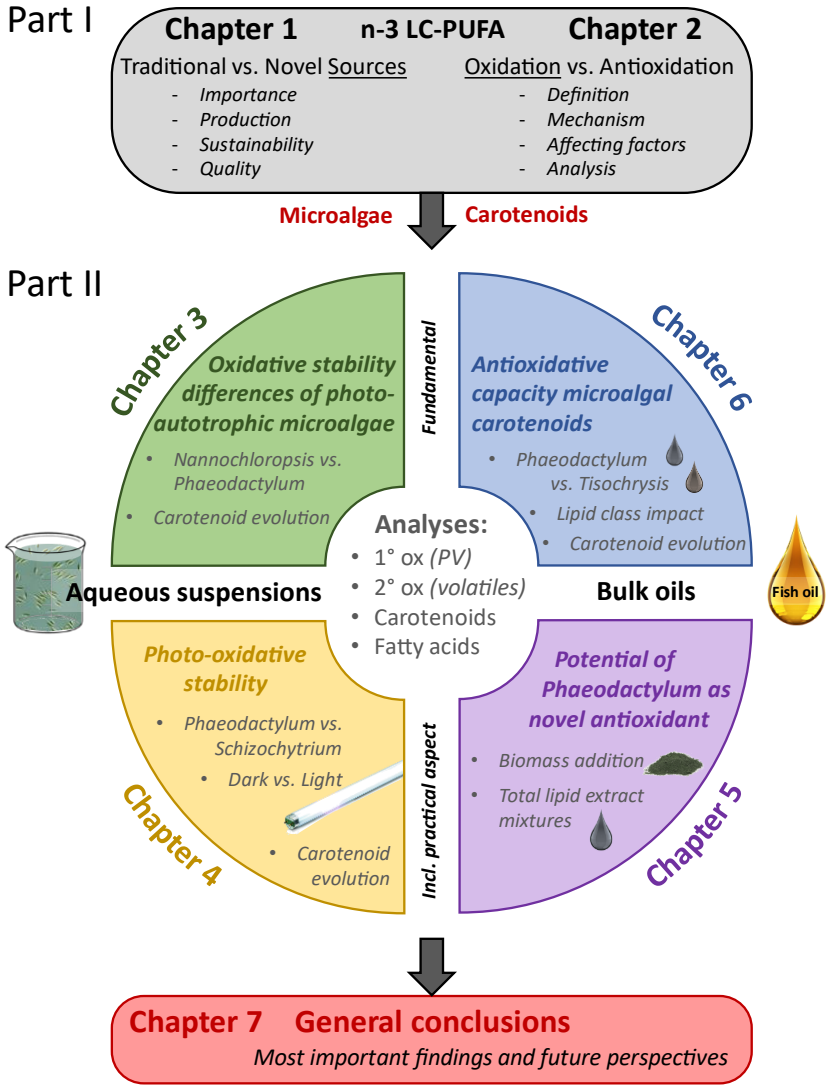


Figure 1: Outline PhD thesis

PART I

LITERATURE REVIEW

CHAPTER 1 - TRADITIONAL AND NOVEL SOURCES OF LONG-CHAIN OMEGA-3 POLYUNSATURATED FATTY ACIDS

Adapted from: Demets, R., & Foubert, I. (2021). Traditional and novel sources of long-chain omega-3 fatty acids. In P. J. García-Moreno, C. Jacobsen, A.-D. M. Sorensen, & B. Yesiltas (Eds.), *Omega-3 Delivery Systems* (pp. 3–23). Academic Press.

1.1. LONG-CHAIN OMEGA-3 POLYUNSATURATED FATTY ACIDS

1.1.1. DEFINITION

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are a class of fatty acids with more than one double bound of which the first one, starting from the methyl end, is located between the third and fourth carbon atom. Based on the number of carbon atoms, a further distinction can be made between long-chain omega-3 polyunsaturated fatty acids (n-3 LC-PUFA), with at least 20 carbon atoms, and short-chain omega-3 polyunsaturated fatty acids (n-3 SC-PUFA), with a shorter carbon chain. The most important n-3 LC-PUFA are eicosapentaenoic acid (EPA, C20:5 n-3), docosahexaenoic acid (DHA, C22:6 n-3) and docosapentaenoic acid (DPA, C22:5 n-3), while α-linolenic acid (ALA, C18:3 n-3) and stearidonic acid (SDA, C18:4 n-3) are well-known n-3 SC-PUFA (Figure 1.1).

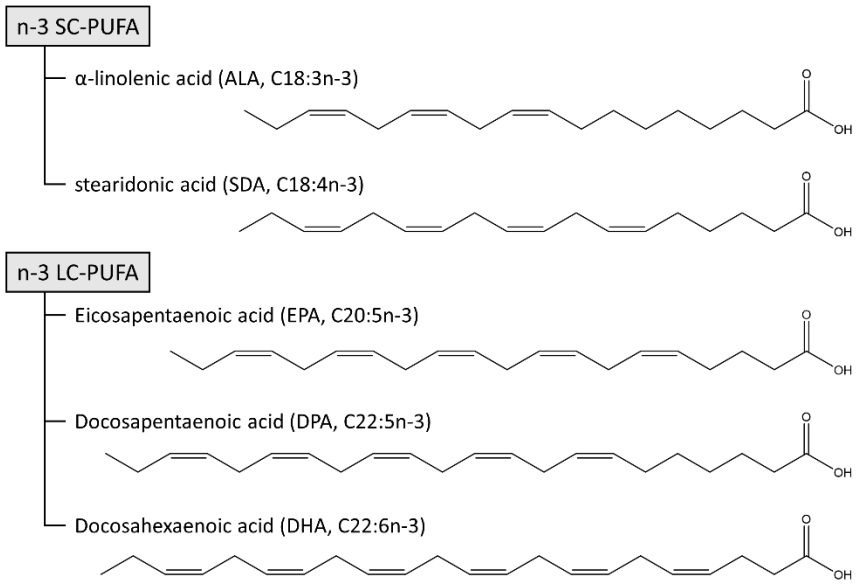


Figure 1.1: Chemical structures of the most important n-3 PUFA

1.1.2. HEALTH BENEFITS

N-3 LC-PUFA, among which especially EPA and DHA, receive a lot of attention due to their generally accepted health benefits. Plenty of research in the form of randomized controlled trials, other epidemiological studies and studies unraveling the underlying molecular mechanisms has been conducted [17].

Especially the beneficial effects related to cardiovascular diseases (CVD) are strongly recognized. N-3 LC-PUFA modify several risk factors (blood pressure, plasma triacylglycerol (TAG) concentration, platelet reactivity and thrombosis, vascular function, cardiac arrhythmias, heart rate variability and inflammation) in such a way that this results in CVD prevention and retardation [18]. Apart from that, n-3 LC-PUFA are often associated with the prevention and treatment of inflammation related diseases like rheumatoid arthritis, inflammatory bowel disease, asthma and psoriasis, driven by the fact that EPA and DHA are precursors of lipid mediators (resolvins, protectins and maresins) with anti-inflammatory properties [19]. N-3 LC-PUFA have also been related to cancer, both in lowering the risk [20] and in improving the therapy, where they could be beneficial for the effectiveness of therapeutic agents [21]. Neurological disorders can also be brought under attention, as n-3 LC-PUFA could help in reducing the burden of psychiatric and depressive illnesses or in preventing neurodegenerative disorders [18].

Next to the above mentioned beneficial health effects, which are mainly ascribed to both EPA and DHA, DHA has a supplemental role in brain and eye function and in infant development [22]. DHA is an important structural component in retina and brain, where it composes around ten percent of the fatty acid composition [23]. Its presence in early life, predominantly maintained by the mother during pregnancy or lactation, is thought to be of great importance for visual and neurological development, as several observational studies and meta-analyses have revealed associations between maternal DHA status and infant and child cognitive, psychological, and behavioral outcomes [22,24]. Apart from EPA and DHA, beneficial roles of DPA as n-3 LC-PUFA are also emerging [18].

1.1.3. CONVERSION FROM ALA

EPA and DHA are semi-essential fatty acids. Like in all vertebrates, they cannot be biosynthesized by the human *de novo*, but they can be produced out of the essential fatty acid ALA due to the presence of several desaturase and elongase enzymes [25]. The consecutive conversion of ALA to EPA and DHA is schematically represented in Figure 1.2 and occurs as follows: first ALA is desaturated to SDA with the help of Δ6-desaturase. A following elongation (by elongase 5) and desaturation (by Δ5-desaturase) leads to the production of EPA, that can be further converted to DPA by another elongation (elongase 5 and 2). Due to the lack of a Δ4-desaturase enzyme the formation of DHA out of DPA takes place via an alternative route called the 'Sprecher pathway', consisting of an elongation (by elongase 2), a desaturation (by Δ6-desaturase) and a peroxisomal β-oxidation [26].

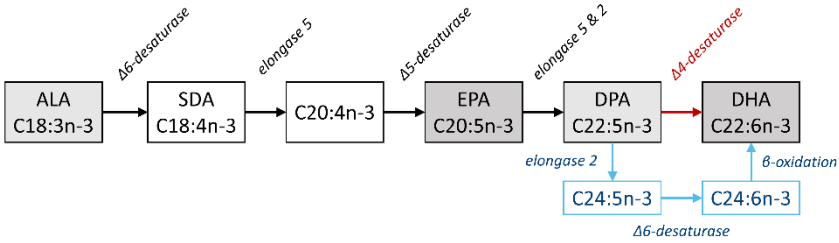


Figure 1.2: ALA to n-3 LC-PUFA conversion (after Monroig & Kabeya [25])

This conversion of ALA to n-3 LC-PUFA is however limited in humans, with reported conversion rates of up to 8% for EPA and up to 4% for DHA [27]. Concerning DHA, multiple studies state even lower values of 0 to 0.5% [28]. Although the reported conversion rates are thus generally low, the exact conversion efficiency depends on several factors. Higher conversion rates are reported for women [29] and the conversion rate seems to decline with age [30]. Furthermore, hormones, genetics, micronutrient status, disease and environmental contaminants have their influence [18,31,32]. The most important reason for the overall low conversion rates however lies in the inhibitory influence of linoleic acid (LA, C18:2 n-6). The desaturase and elongase enzymes, that are used in the conversion of ALA to n-3 LC-PUFA, share their activity with omega-6 polyunsaturated fatty acids (n-6 PUFA) where LA is converted to arachidonic acid (AA, C20:4 n-6) in an analogous pathway. As Western diets are characterized by a low n-3:n-6 intake ratio (1:15-20) the conversion of LA to AA rather than the one of ALA to EPA and DHA is promoted [33]. ALA that is not converted to n-3 LC-PUFA is predominantly being used as energy source (by β-oxidation) or being stored in adipose tissue [34,35].

1.1.4. IMPORTANCE OF SOURCES

The multitude of health benefits associated with n-3 LC-PUFA in combination with the low conversion rates from ALA imply the importance of preformed sources of EPA and particularly DHA in the human diet [36]. In what follows, the most important sources of n-3 LC-PUFA are discussed in terms of production, sustainability and quality. First, fish will be discussed as the most traditional source. The shortage of n-3 LC-PUFA created by a rising population and an increased health benefit awareness on the one hand and sustainability issues resulting from overfishing a finite fish stock on the other hand, however raise the importance of novel n-3 LC-PUFA sources [37,38]. These novel sources, of which alternative marine resources, microalgae and transgenic plants are the most important ones, will be thoroughly discussed thereafter.

1.2. TRADITIONAL SOURCES

Fish is, and has by far been, the most important source of n-3 LC-PUFA. It covers almost 90% of our supply, a vast majority that is not only provided by fish as a food product on its own, but also by fish oil and fish meal [4]. This distinction between food fish and fish derivatives will be made in the following discussion about fish as the most traditional n-3 LC-PUFA source. Although they are not explicitly mentioned, shellfish are hereby also referred to as 'fish'.

1.2.1. FISH

1.2.1.1. IMPORTANCE

Although fish are, just like humans, unable to produce n-3 LC-PUFA *de novo*, they are considered as a rich source, providing most of the EPA and DHA in diets of people around the world [32]. The EPA and DHA fish contains are also derived from their nourishment and can be traced back to microalgae (phytoplankton) as the primary producer. These aquatic micro-organisms, which can be found in both marine and freshwater environments, synthetize n-3 LC-PUFA at the base of the aquatic food web. With a transfer through several trophic layers (zooplankton feeding on microalgae, fish feeding on zooplankton etc.) they are responsible for the majority of n-3 LC-PUFA present in aquatic organisms [11].

The n-3 LC-PUFA content in fish shows great variations, differing with two orders of magnitude [39]. Table 1.1 gives an overview of the EPA + DHA content of common fish and shellfish species. Especially oily fish like sardines, salmon, mackerel and herring seem to be the best sources with a content of around 1 to 2 g/100 g [40]. Nonetheless, species that are low in fat like shrimp and trout are also good sources, making fat content only moderately predictive for their quality as n-3 LC-PUFA source [32]. The large amount of variation can be assigned to both phylogenetic and ecological factors. Gladyshev et al. [39] performed a meta-analysis on phylogeny, trophic level, habitat, size and movement as influencing factors, and found that especially marine planktivorous fast swimmers are characterized by high n-3 LC-PUFA contents. This is in contrast to the greater believe of high trophic levels and cold environments being determinant for a high n-3 LC-PUFA content [41,42].

Table 1.1: EPA + DHA content of common fish and shellfish species (after Czerner et al. [43], Mahaffey et al. [32] and Puri et al. [40])

EPA + DHA content of common fish and shellfish species			
Species	**EPA + DHA (g/100 g)**	**Species**	**EPA + DHA (g/100 g)**
Anchovy	1.15	Perch	0.30-0.32
Catfish	0.17-0.28	Pike	0.13-0.14
Clam	0.24-0.28	Pollock	0.26-0.54
Cod	0.15-0.24	Salmon	1.59-2.14
Crab	0.04-0.41	Sardine	0.98
Eel	0.18	Scallop	0.18-0.27
Flatfish	0.02-0.50	Sea bass	0.49-0.76
Haddock	0.18-0.23	Shark	0.22-0.84
Herring	2.01	Shrimp	0.31-0.39
Lobster	0.36-0.48	Swordfish	0.58-0.81
Mackerel	1.79-1.84	Trout	0.58-0.93
Mussel	0.35-0.78	Tuna	0.32-0.63
Oyster	0.35-0.44		

1.2.1.2. PRODUCTION

The global fish production was estimated at 178 million tons in 2020, of which 157 million tons (89%) were devoted to human consumption. The per capita fish consumption in 2020 was estimated at 20,2 kg. Compared to a per capita fish consumption of 9 kg in 1961, global fish consumption and production has shown a great increase caused by technological developments, rising incomes, reductions in loss and waste and an increased awareness of fish consumption related health benefits [3,44]. As a growing world population puts pressure on an inelastic fish stock that has been plateauing since the 1990's [36], aquaculture or fish farming has grown to be a major shareholder of the world fish production with 87.5 million tons (49.2%) of the global fish production being produced by aquaculture [3]. Since 2016, aquaculture has even surpassed capture fisheries as the main source for human consumption [44].

1.2.1.3. SUSTAINABILITY

The global fish production, captured as well as farmed fish production, has a large environmental impact. As currently approximately one third (35.4%) of the wild fish stocks are being exploited in a biologically unsustainable way, and only 7.2% of the remaining 64.6% that are fished sustainably offer possibilities for growth,

the global fish stock is declining [3,45]. The increasing demand for fish products made it necessary to resort to fish farming [46]. However, while aquaculture seems to relieve the pressure on catching fisheries, this is not always the case. On the one hand fish farming has a negative impact on wild fish by habitat modification and pollution of the natural water body sections they use [2,11,47]. On the other hand aquaculture is directly dependent on the input of wild fish in their feed as a source of proteins and n-3 LC-PUFA. The input of n-3 LC-PUFA is not only important for the nutritive quality of farmed fish as a food product but also for the health status of the farmed fish on its own. Initially, this resulted in the input of wild fish feed (predominantly as fish oil or fish meal) being higher than the obtained biomass, making fish farming a net fish user rather than a net fish producer [2]. Therefore fish farms increasingly switched to more sustainable feeds, largely based on terrestrial agriculture (e.g. rapeseed oil), enabling the enormous growth in production that was mentioned above [4]. While this dietary substitution is most challenging for carnivorous species like salmon and trout, which still use more or equally as much fish biomass as is consumed, most aquaculture species are currently considered net fish producers [48,49].

1.2.1.4. QUALITY

While the quality of wild fish as an n-3 LC-PUFA source depends on the discussed variable content, the replacement of fish oil or fish meal with terrestrial alternatives in the aquaculture sector is having a great impact on the quality of farmed fish. Due to the fact that these alternatives lack the presence of n-3 LC-PUFA, their implementation provoked a reduction in the EPA and DHA content of farmed fish [38]. The n-3 LC-PUFA content of farmed salmon for example has halved when comparing their fatty acid composition of 2015 with the one from 2006 [50]. This means that the consumption of farmed fish would have to increase to have an equally beneficial health effect. To ensure the nutritive quality of farmed fish, future aquaculture production will therefore need to resort to other n-3 LC-PUFA sources, like the novel sources that will be discussed below, in their feed [51].

The n-3 LC-PUFA content of fish as a raw food product is not the only thing that should be kept in mind considering its nutritive value. Both storage and processing can have a significant impact by causing lipid oxidation [46]. This undesired chemical process, which will be extensively covered in Chapter 2 ('Oxidation of n-3 LC-PUFA'), is well-known as a major factor responsible for quality loss during storage of seafood. Apart from the general factors affecting lipid oxidation

(temperature, oxygen availability etc.), the oxidation rate is dependent on species and muscle type, with skin and dark muscle being more susceptible than white muscle [52]. Disregarding the storage, the influence of several cooking methods was studied for a variety of species. While some studies showed that cooking caused a decrease in EPA and DHA content, other showed them to be unaffected [53]. Only a limited number of these studies however took the problem of lipid oxidation into consideration by also measuring real oxidation markers such as peroxide value (PV) and thiobarbituric acid reactive substances (TBARS), showing a small increase after cooking [54,55].

Considering the quality of fish as a source, other components than n-3 LC-PUFA are of importance as well. On the one hand, fish contains a lot of other beneficial nutrients of which essential proteins, iodine, selenium and vitamin A, D and E are the most important ones [42]. On the other hand, fish is known for the biomagnification of lipophilic organic contaminants. Especially methylmercury, known for its ability to cause neurological damage, is responsible for fish consumption to be perceived as unsafe. Certainly in the case of fish consumption during pregnancy, when the brain of the fetus is still developing, the risk of a harmful methylmercury intake is high. However, the intake of sufficient n-3 LC-PUFA just then is of importance as well. Luckily, it seems possible to choose fish both high in n-3 LC-PUFA and low in methylmercury, as both components have a different retention pattern. Species like mackerel, salmon and sardines with a low-to-moderate methylmercury content have a high n-3 LC-PUFA content [32]. According to Rizzo & Baroni [46] methylmercury is less of a problem in farmed fish, but other lipophilic contaminants (PCBs and dioxins) could be found in higher concentrations and their presence should therefore be taken into account as well.

1.2.2. FISH DERIVATIVES

1.2.2.1. IMPORTANCE

Despite the fact that the vast majority of fish production is devoted to direct human consumption, the fish derivatives fish oil and fish meal are important to acknowledge as well. They have their main use as feed ingredient in aquaculture. Fish meal mainly serves as a protein source, while fish oil rather provides the n-3 LC-PUFA. Although n-3 LC-PUFA are stated to be present in fish meal [56], especially fish oil is a rich source of n-3 LC-PUFA and therefore deserves the main focus in this regard. The n-3 LC-PUFA content of fish oil again depends on the fish species being used as a raw material, and can go up to 35% [57]. Next to their use as

feed ingredient in aquaculture, fish oil is being used in some pig and poultry animal feeds [4,58]. Furthermore, fish oil can be used for a more direct human consumption: either by implementing fish oil in the formulation of certain food products (e.g. margarine enriched with n-3 LC-PUFA) or by consuming it as a food supplement (nutraceutical) (e.g. cod liver oil) [57].

1.2.2.2. PRODUCTION

Of the 178 million tons of fish produced in 2020, 16 million were devoted to the production of fish oil and fish meal. According to the Marine Ingredients Organisation 75% of the produced fish oil is being used in aquaculture feeds and 21% goes into nutraceuticals [3,59]. The major fish species used for the production of fish oil is anchovy: share estimations range between 30 and 50%. Together with sardine, mackerel and herring, they represent around three quarters of the fish raw material that is being used [60,61]. The fish oil production process can be divided into two major parts: extraction and refining. For the extraction, the wet pressing method, in which raw fish is being cooked and pressed, is the most used. A following filtration or centrifugation step results in the production of crude fish oil and fish meal. Alternative methods for obtaining crude fish oil are supercritical fluid extraction or proteolytic extraction methods using enzymes. The refining consists of several steps and aims to remove several unwanted compounds. Degumming results in the loss of phospholipids (PLs), neutralization removes the free fatty acids and bleaching ensures the elimination of pigments and contaminants, resulting in a clear oil. A final deodorization step removes the unwanted odor compounds. If desired the n-3 LC-PUFA can be further concentrated using several methods (chromatography, distillation, low-temperature crystallization, urea complexation) to obtain high-quality nutraceuticals [57]. Further information on the extraction, refining and concentration of n-3 LC-PUFA oils can be found in the book chapter of Kulås [62].

1.2.2.3. SUSTAINABILITY

As fish oil is produced from captured fish, the problem of overfishing again comes to mind. Because of the limited fish stock, fish oil can be considered as a finite resource as well. The reliance on it by a growing aquaculture industry, is therefore an unsustainable practice [2]. Apart from the usage limitation by replacing fish oil with terrestrial oils that was discussed above, fish oil is increasingly being used at specific stages of production with an implementation in hatchery, broodstock and finishing diets rather than in grower diets [4]. Another tendency is the increasing by-product processing, where also by-catch and trimmings are being used to

produce fish oil instead of solely starting from whole feed grade fish [59]. 48% of the raw material volume for fish oil production originates from by-products [3].

1.2.2.4. QUALITY

Because of its high n-3 LC-PUFA content, fish oil is considered an n-3 LC-PUFA source of great quality. However, this high degree of unsaturation goes along with a high susceptibility to lipid oxidation. As optimizing processing, storage and packaging conditions do not suffice in limiting this undesired process, antioxidants are usually added to obtain a quality product with a longer shelf-life. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are well-known, effective synthetic antioxidants, but as they suffer a negative perception, natural antioxidants like rosemary extracts are increasingly being used [57,63]. Lipid oxidation, in general and of fish oil in particular, will be further elaborated in Chapter 2.

Due to the lipophilic nature of the organic contaminants that were discussed for food fish as a source, they also pose a risk for consuming fish oil or using fish oil as a feed source. Therefore, effort is being made to implement cost-effective decontamination solutions in the refining process of fish oil production. Physical adsorption on activated carbon seems to be an adequate method [64], but other techniques, such as steam deodorization, short path distillation and supercritical CO_2 extraction, are being considered as well [65].

1.3. NOVEL SOURCES

Novel n-3 LC-PUFA sources, i.e. n-3 LC-PUFA sources other than fish, can be divided into three different groups. Firstly, other, sustainably fishable, yet currently underexploited marine resources that are part of the existing marine n-3 LC-PUFA stock can be addressed upon. Secondly, microalgae, the primary n-3 LC-PUFA producers, can be mass cultured to generate n-3 LC-PUFA *de novo* in a natural way. A third option is not to cultivate the microalgae themselves, but to transfer their n-3 LC-PUFA production encoding genes into terrestrial crops, resulting in genetically modified plants that can subsequently be grown and processed with the agricultural infrastructure that is already at hand. Similarly to fish and fish derivatives, all three approaches will be discussed in terms of importance, production, sustainability and quality.

1.3.1. ALTERNATIVE MARINE SOURCES

1.3.1.1. IMPORTANCE

While most fish is currently being overexploited, resulting in a declining fish stock [66], several alternative marine resources that are also rich in n-3 LC-PUFA could be targeted as well. The most important ones in this regard are marine invertebrates (more specifically copepods and krill), mesopelagic fish and by-products of seafood processing [67].

Copepods and krill are both small crustaceans that belong to the zooplankton, a lower trophic level in the marine food web. With a size ranging from 1 to 8 mm copepods are smaller than krill of which the size varies from a few millimeters to 15 cm. Although both can be rich in n-3 LC-PUFA [68,69], this size difference causes krill to receive more attention as this goes along with a larger catch potential. Especially Antarctic krill (*Euphausia superba*) is of importance, because it offers a high n-3 LC-PUFA content (with reported values of up to 60% of the fatty acid profile), a large size and an abundant availability [67].

Mesopelagic fish are fish that live in the mesopelagic zone enclosing the water mass between depths of 200 and 1000 meters. Although they are fish, they are considered a novel n-3 LC-PUFA source since they are currently not harvested and knowledge in terms of distribution, abundance and nutritional value is still limited. Alvheim et al. [70] recently reported n-3 LC-PUFA contents of 6 different mesopelagic species with values varying from 0.96 to 2.44 g/100 g wet weight.

Especially the interest in the exploitation of certain myctophids (lantern fish) is growing [70].

By-products from seafood processing are increasingly being used as an n-3 LC-PUFA source and could therefore be regarded as novel source as well. It comprises viscera, heads, backbones, cuts and seafood that is rejected from processing [67]. An interesting by-product is the viscera from squid, a seafood that is especially popular in Asian countries like Japan and Korea [71]. Not only does the squid viscera make up a large part of the squid body weight (more than 20%) and does it contain a higher lipid content than the viscera of fish, the lipid fatty acid profile also predominantly consists of n-3 LC-PUFA (40%) making it a source of great potential for the production of n-3 LC-PUFA rich oils [72,73].

1.3.1.2. PRODUCTION

The commercial harvest of marine invertebrates is currently limited to Antarctic krill and small quantities (ca. 1000 tons) of the copepod *Calanus finmarchicus*. The small size or small densities (due to lack of schooling behavior) of several other species make it economically challenging for them to be harvested by the current fishing techniques [4,67]. With an amount of 300,000 tons, the annual catch of Antarctic krill is far less than the precautionary catch limit of 5.6 million tons set by the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) [67]. This demonstrates that Antarctic krill, in contrast to fish is an underutilized source of n-3 LC-PUFA [74]. Important to remark is that most of the harvested krill is used as aquaculture feed, making it a predominantly indirect source of n-3 LC-PUFA for humans [75]. The direct human consumption mainly occurs via krill oil, which has been approved as a novel food product since 2009 [76]. Several technologies are used for the production of krill oil (solvent extraction, nonsolvent (mechanical) extraction, super- or subcritical fluid extraction, and enzyme-assisted extraction) which are all challenged by the fact that krill contains a lot of proteolytic enzymes causing a fast deterioration [74].

Mesopelagic fish are currently not harvested. While they have been in the past following their discovery, their fishing decreased due to the fact that more accessible fish species were available at the time [67].

Just like for krill, the predominant use for aquaculture feed is also valid for seafood by-products [72]. As stated in section 1.2.2., the current fish oil production (which is mainly used for aquaculture feed) originates for 48% from by-products [3]. Similarly

to krill oil, squid oil can be used for direct human consumption as it also has been approved as a novel food [77].

1.3.1.3. SUSTAINABILITY

The usage of alternative marine resources is trying to enlighten the sustainability issues endangering the current fish harvest, and should therefore be sustainable. However, attention should be paid not to increase the harvest of these novel sources to such an extent that they would face the same problem of overfishing. This is especially true in the case of marine invertebrates. Since they belong to a lower trophic level in the marine food-web, unsustainable exploitation would have a negative impact on the higher trophic species that depend on them as a food source as well [78]. Nonetheless, Olsen et al. [67] stated that a properly regulated exploitation of the full range of trophic levels of the marine ecosystem could be more sustainable and have a higher biomass outtake than the current fisheries.

1.3.1.4. QUALITY

Although dependent on seasonal change, the discussed alternative marine sources can all contain a considerable amount of n-3 LC-PUFA [67]. Similarly to fish, the quality of alternative marine resources should however be looked at in a broader way than n-3 LC-PUFA content alone. Regarding the lipid class composition, krill shows an advantage as it is characterized by a large amount of PLs (from 40-80% of total lipids) to which the n-3 LC-PUFA are predominantly bound, resulting in a higher bioavailability [74,79]. Copepods and mesopelagic fish on the other hand contain a considerable amount of wax esters rather than TAGs possibly limiting their use [67,80]. Lipophilic contaminants could be less of a problem in the discussed sources: the levels in marine invertebrates are lower due to their lower trophic level, and for squid a shorter life cycle makes it less likely to accumulate toxins [73]. Also the problem of oxidative instability seems to be lower. Krill oil is characterized by the presence of astaxanthin [74] which, by acting as an antioxidant, causes a higher oxidative stability [81]. Conducting a storage experiment of 400 hours at 37 °C, Cho et al. [82] showed that squid oil from squid viscera could also possesses a favorable oxidative stability, which was attributed to the presence of small amounts of PLs. However, it should be noted that the oxidative stability of PL rich species cannot be assessed by traditional methods such as PV and anisidine value (AV) alone. The amine containing compounds present in these sources have been shown to rapidly react with lipid oxidation products, causing an underestimation of the degree of oxidation when only considering these. Therefore, other methods, such as gas chromatography

coupled with mass spectrometry (GC-MS) (which will be discussed in Chapter 2), should be regarded to as well [83], which was the case for the study of Ryckebosch et al. [81], but not for the study of Cho et al. [82].

1.3.2. MICROALGAE

1.3.2.1. IMPORTANCE

As stated above, the production of the majority of n-3 LC-PUFA present in aquatic organisms can be related to microalgae as primary producer. Cultivating them should therefore be an interesting approach in the search for a novel n-3 LC-PUFA source. However, it has to be pointed out that not all microalgal species are capable of producing these fatty acids. Microalgae comprise a large group of unicellular organisms featured by an enormous diversity. They differ not only in n-3 LC-PUFA producing ability, but also in size (ranging from a few micrometers to a few hundred micrometers), phylogeny (although mainly part of eukaryotic classes, the prokaryotic cyanobacteria are considered microalgae as well), appearance (individually, in chains or in groups), composition and way of satisfying energy and nutrient needs [84]. In terms of energy and nutrient supply, a distinction can be made between photoautotrophic, heterotrophic and mixotrophic microalgae. Similar to land plants, photoautotrophic species meet their energy needs by photosynthesis, converting inorganic carbon dioxide, nitrogen, sulfur and phosphorus into the biomass required for their growth and development, using light as the sole energy source. Heterotrophic species on the other hand depend on an organic carbon source, enabling them to grow in the absence of light. Mixotrophic species can simultaneously perform photosynthesis and catabolize organic compounds [85].

For microalgae as a novel n-3 LC-PUFA source, the focus should be on the microalgal species rich in n-3 LC-PUFA. Table 1.2 gives an overview of the most interesting ones comprising both photoautotrophic and heterotrophic microalgae [12]. It is clear that lipid as well as n-3 LC-PUFA content not only show a great variation between, but also within the different species, which can be (partially) explained by the influence of environmental factors strongly influencing the lipid metabolism. These include both general conditions (temperature, light, salinity and pH) and nutrition (availability of carbon dioxide, nitrogen, sulfur and phosphorus in the case of photoautotrophic growth or organic carbon sources in the case of heterotrophic growth) [86]. The impact of nutrient availability arises regarding the growth phase of the microalgae: most species have their highest

EPA or DHA content in the stationary phase following exponential growth due to nitrogen limitation [87].

Table 1.2: Total lipid (% of dry biomass), EPA (% of total fatty acids) and DHA (% of total fatty acids) content of different n-3 LC-PUFA rich microalgal species (from Gheysen et al. [12])

Phylum	Species	Total lipids (% of dry biomass)	EPA (% of total fatty acids)	DHA (% of total fatty acids)
Bacillariophyta	*Chaetoceros*	9-40	8-22	0.03-5
	Phaeodactylum	15-40	2-36	0-3
	Skeletonema	3-25	13-36	1-6
	Thalassiosira	9-26	11-17	1-5
Chlorophyta	*Tetraselmis*	6-26	4-11	0-0.1
Cryptophyta	*Cryptomonas*	12-22	3-25	2.5-10
	Rhodomonas	7-19	8-18	4-9
Dinoflagellata	*Crypthecodinium*	30-50	0-1	47-97
Haptophyta	*Diacronema*	7-36	5-29	4-19
	Tisochrysis	7-33	1-27	1-40
Heterokontophyta	*Nannochloropsis*	16-68	9-38	0.03-3
	Schizochytrium	50-77	0-2	20-53
Rhodophyta	*Porphyridium*	11-18	2-38	0-0.2

In terms of structure, eukaryotic microalgae are comparable with plant cells. They house different cell organelles: nucleus, endoplasmic reticulum, ribosomes, Golgi apparatus, mitochondria and vacuole [84]. Photoautotrophic species contain a chloroplast performing photosynthesis, while heterotrophic ones do not [88]. Embedded within the cytosol these organelles are surrounded by a cell membrane and a cell wall. The cell membrane serves as a selective barrier. The cell wall has a higher porosity and shows strong structural variation between different species: from a rigid structure consisting of multiple layers to a complete absence [84,89,90]. Microalgal lipids are an important part of both cell and cell organelle membranes, but can also be stored in lipid bodies within the cytoplasm. Acting as energy reserves, these lipid bodies are generally formed upon stress conditions and mainly consist of neutral lipids [91]. Membrane bound lipids (like the ones in the thylakoid membranes of the chloroplast) on the other hand consist of more polar lipids [88]. Since a considerable amount of n-3 LC-PUFA, ranging between 20 and 40%, is bound to the polar PL fraction [92], this implies that n-3 LC-PUFA are largely present in the membranes.

1.3.2.2. PRODUCTION

The production process of microalgae can be divided into four different steps: cultivation, harvesting, drying and biomass processing [12]. Cultivation consists of growing the microalgae to acceptable densities. For photoautotrophic species this can be done in either open raceway ponds or in closed photobioreactors. While the latter have a higher investment and operational cost, and are harder to scale-up, they are characterized by a better protection against contamination, a larger controllability and higher cell densities [93]. For heterotrophic species, which are grown in fermenters, the achievable cell densities are ten- to hundredfold higher due to their light independency [94]. Harvesting aims to obtain a dense, wet algal paste by separating the microalgal cells from the culture medium. For this several techniques can be used (centrifugation, sedimentation, filtration, flocculation and flotation), which are all challenged by the small size of the microalgal cell, the limited density difference between cell and culture medium, and possibly low concentrations of cells in the culture medium, causing harvesting to be an energy-intensive step [13]. These limitations are less prominent for heterotrophic cultivation due to the higher cell densities. The subsequent drying step, applicable on both photoautotrophic and heterotrophic species, also has a high energy-intensity [12]. Drying is usually done by either spray or freeze drying. Although it is not always implemented in the production process, it might be necessary to obtain a stable product since the storage of the wet microalgal paste could, depending on the species, result in lipolytic degradation by endogenous lipases [95]. Similarly to the other sources discussed above (fish, krill and squid), the obtained biomass can be further extracted to obtain an n-3 LC-PUFA rich oil. Again, several techniques can be used (solvent or supercritical), of which the efficiency can be improved by combining extraction with a cell disruption technique (either mechanical, physical, enzyme-assisted or chemical) [96,97].

High capital and operational costs of cultivating microalgae accompanied by the high energy-intensity of subsequent harvesting and drying cause microalgal biomass to be an expensive product [85]. This high cost, which is only raised even more by a possible oil extraction, is limiting its use in the aquaculture sector [98]. Whereas fish oil and krill are predominantly used as feed in aquaculture settings, microalgae are currently more of a niche product for direct human consumption or for feeding high-value marine species [4]. Regarding human consumption, the heterotrophic species *Schizochytrium* and *Crypthecodinium cohnii* are dominating. Their oil is being used as an n-3 LC-PUFA source in the infant milk formula market and in the beverage and supplement industry in general.

Although both species, and as a result their oil, are generally characterized by the presence of DHA rather than EPA, an oil derived from a specific *Schizochytrium* strain with both DHA and EPA (ratio 2:1) is being produced as well [99]. The fact that no photoautotrophic n-3 LC-PUFA rich species are used for direct human consumption is partially caused by a higher production cost due to the lower achievable cell densities, as well as by the European Novel Food Regulation which currently only authorizes *Odontella aurita* and *Tetraselmis chui* in this regard [100]. Applications for *Nannochloropsis* and an EPA-rich oil from *Phaeodactylum* have been submitted [101–103]. For the feeding of high-value marine species on the other hand, photoautotrophic microalgae are used. *Tisochrysis* and *Nannochloropsis* are used as feed for rotifers [40,104], which can subsequently be used to nurture certain larval marine crustaceans and fish species [4,105]. Regarding the quantity of production, only a small part of the microalgae production market is aimed at n-3 LC-PUFA rich species: whereas the total production in 2018 amounted 87,000 tons [44], *Crypthecodinium cohnii* and *Schizochytrium* accounted for only 500 and 20 tons respectively [101].

1.3.2.3. SUSTAINABILITY

While using fish or alternative marine resources as an n-3 LC-PUFA source predominantly relies on the usage of the naturally available n-3 LC-PUFA, cultivating n-3 LC-PUFA rich microalgae generates a new supply. Moreover, the cultivation of microalgae has several advantages compared to traditional crops: it is characterized by a higher growth rate and therefore a higher biomass productivity (10 to 50 times higher than terrestrial plants), there is no need for high quality agricultural land and the freshwater consumption is lower since brackish or sea water may be used [85]. This greatly promotes the usage of microalgae from a sustainability perspective. This is especially true for photoautotrophic microalgae, which do not rely on the usage of an organic carbon source [106]. However, the high energy-intensity of producing consumable microalgal biomass or oil should be kept in mind. Nevertheless, Schade et al. [107] recently compared the environmental impact of using *Nannochloropsis* and *Phaeodactylum* as an n-3 LC-PUFA source with wild captured or farmed fish using life cycle assessments, and found that microalgal biomass has a lower environmental impact than fish fillet from both capture and aquaculture production if the burden of CO_2 production is neglected. Implementing CO_2 production showed similar to lower impacts than aquaculture fish. Furthermore, technological advances in the microalgal production chain are thought to further decrease the environmental impact [14].

1.3.2.4. QUALITY

Microalgal biomass is of high nutritional quality. Apart from n-3 LC-PUFA they are a source of both macronutrients (proteins, carbohydrates and other lipids) of which the amount is comparable to common food products, and beneficial micronutrients such as carotenoids, vitamins and phytosterols. A large diversity in composition between different algal species offers the possibility of a directed choice depending on application [13]. Although the composition is greatly influenced by environmental factors, these can be controlled during cultivation leading to a more constant composition than is the case for fish [108]. As was mentioned in 1.3.2.1., a considerable amount of n-3 LC-PUFA is bound to the PL fraction [92]. As it has been suggested (in krill oil studies) that PLs have an intrinsically higher bioavailability than TAG, this could also contribute to the nutritional quality [79].

A distinction should be made between microalgal biomass and microalgal oil. For direct human consumption the usage of whole cell biomass would result in the intake of all nutrients, whereas the oil would only provide the extracted lipophilic nutrients. However, for the biomass the microalgal cell (wall) could act as a physical barrier obstructing the uptake of nutrients stored inside the cell. Especially for microalgal species with a strong cell wall such as *Nannochloropsis* [89], supplemental processing causing cell (wall) disruption is necessary to improve bioaccessibility [109]. In the case of using microalgae in fish feeds, the digestibility of the cell wall should also be considered [98]. The presence of carbohydrates limits the inclusion of microalgal biomass in the feed of carnivorous species such as salmon, leading to a preference for the use of microalgal oil [17]. *Schizochytrium* biomass has however proven to be a quality fish oil substitute [98].

Another quality distinction can be made in terms of oxidation. Whereas the oxidative stability of heterotrophic microalgal species such as *Schizochytrium* is limited, certain photoautotrophic species show a high resistance against lipid oxidation, which can be attributed to the presence of endogenous antioxidants [8,110]. This finding will be further discussed at the end of Chapter 2 (after considering general lipid oxidation and antioxidation aspects), and is of major interest for the current PhD research.

1.3.3. TRANSGENIC PLANTS

1.3.3.1. IMPORTANCE

Rather than exploiting the n-3 LC-PUFA producing capacity of microalgae by cultivating them, another approach is to transfer their genes encoding for n-3 LC-PUFA production to oilseed crops who, like all terrestrial organisms, lack the presence of such genes. In this way the well-established infrastructure for the production of vegetable oils dominating the world oil production, that is already at hand, could be used instead of developing new technologies for the large scale production of microalgal biomass or oil [111]. Starting from ALA or oleic acid (OA), two fatty acids that do occur in terrestrial crops, the incorporation of several non-native sequential enzymes is required: at least three or five for the conversion from ALA to respectively EPA or DHA (see Figure 1.2), and five or seven starting from OA as the conversion from OA to ALA requires two more enzymes. The evolution of developing n-3 LC-PUFA rich oilseed crops however started with the development of transgenic crops rich in SDA, an intermediate in the metabolic pathway from ALA to EPA. This was established in both soybean and canola leading to an oil containing respectively 18-28% and 16-23% SDA [112,113]. Thereafter the possibility of producing DHA by gene incorporation in terrestrial plants was demonstrated in *Arabidopsis thaliana* as a model organism [114]. This was followed by the development of two n-3 LC-PUFA rich oilseed crops [111]. First, transgenic *Camelina sativa* arose of which the oil contained either 16-17% EPA or 4-5% EPA and 4% DHA [115,116], and more recently a DHA rich canola oil was developed showing fish-oil like DHA levels (9-11%), and thereby the highest observed DHA content in seed oils thus far [117]. For the sake of completeness it should be mentioned that not only the metabolic engineering of oilseed crops, but also of oleaginous micro-organisms has been aimed at. The metabolically engineered yeast strains of *Yarrowia lipolytica* have been the most successful in this regard, resulting in a strain producing 15% EPA on dry weight basis [111,118]. With the production of this yeast occurring in fermenters, similarities can be seen with the heterotrophic microalgae discussed above.

1.3.3.2. PRODUCTION

While the production of both n-3 LC-PUFA rich camelina and canola oil has been limited to field trials, both show a great potential for the large-scale production of *de novo* n-3 LC-PUFA. Camelina can be cultivated on low quality agricultural land and still show a high oil yield (800 kg/ha) in unfavorable weather conditions. Moreover, the seeds have a high oil content of 35-45% [115]. Both advantages are

also valid for canola, that shows an oil yield of up to 1800 kg/ha with seeds containing 40-45% oil. After soybean, canola is the oilseed crop that is produced the most [117].

While the infrastructure for large scale production is at hand, the use of transgenic plants is however not well accepted worldwide, especially not in Europe [57]. As consumers tend to prefer natural n-3 sources over genetically modified organisms (GMOs) [119], the public opinion towards GMOs would need to change before a global commercialization is possible. Also, regulations would need to be revised as organisms generated through gene editing are in practice currently excluded from practical and commercial opportunities in the EU [120].

1.3.3.3. SUSTAINABILITY

Similarly to cultivating microalgae, growing transgenic plants generates a new supply of n-3 LC-PUFA, which is more sustainable than overexploiting a finite fish stock. Regarding n-3 LC-PUFA rich canola, the transgenic oilseed crop with the highest DHA content so far, 1 ha would be equivalent in terms of n-3 LC-PUFA to 10,000 fish [121]. Differently stated, the production of 2,5 million ha, which amounts to 2% of the global cultivation of oilseed crops, would be equivalent to the currently harvested DHA amount of all fish oils [122]. Differing from cultivating microalgae, the use of arable land would be needed, exerting pressure on the existing agricultural land. From a sustainability point of view it would however be ideal to switch part of the vegetable oil production from n-6 PUFA producing crops to n-3 LC-PUFA producing crops [111]. Moreover, in the regard of sustainable land usage of oilseed crops, interesting research is being conducted on using genetic modification to stimulate oil production in non-seed parts like stems and leaves for obtaining a higher oil yield [123,124]. Attention should however be paid to the possible environmental impact of GMOs. The introduction of novel genes (such as the ones encoding for EPA or DHA production) in the terrestrial ecosystem, could result in an unintended ecological impact. The physiology and ecology of land-based insects (and their consumers) not being used to consuming n-3 LC-PUFA, could be altered and a gene flow to wild relatives could occur [125].

1.3.3.4. QUALITY

Next to successful field trials for both transgenic camelina and canola demonstrating their potential as a novel oilseed crop with a high n-3 LC-PUFA content [115,117], studies on the incorporation of transgenic camelina oil into fish feeds have been conducted. Since no negative impacts on fish growth or health

were observed, and the incorporation led to clearly increased n-3 LC-PUFA contents, it could be a suitable alternative for fish oil [17,126]. Whereas the problem of lipophilic contaminants characterizing marine environments is avoided when using transgenic terrestrial crops, a possible quality problem that should be examined is lipid oxidation. Since specific endogenous antioxidants are thought to have a protective role in krill and photoautotrophic microalgae, their absence in terrestrial crops might cause a low oxidative stability. Petrie et al. [117] reported a long-term stability of transgenic canola n-3 LC-PUFA during a storage experiment of 6 months at 32°C. However, not the oil but the seeds were stored, and no oxidation products were analyzed.

1.3.4. ADVANTAGES AND LIMITATIONS

All novel n-3 LC-PUFA sources offer opportunities and pose different challenges. Table 1.3 summarizes both advantages and limitations of the three possibilities discussed above.

Table 1.3: Advantages and limitations of novel n-3 LC-PUFA sources

Source	Advantages	Limitations
Alternative Marine Sources	✓ Lower content of lipophilic contaminants *(krill, copepods, squid)* ✓ Bioavailability *(krill)* ✓ Oxidative stability *(krill)*	✗ Risk of overfishing ✗ Current fishing techniques ✗ Fast deterioration *(krill)* ✗ Wax esters *(mesopelagic fish, copepods)*
Microalgae	✓ New supply ✓ Sustainability advantages over traditional crops ✓ High nutritional quality ✓ High cultivation control ✓ Bioavailability *(oil)* ✓ Oxidative stability *(photoautotrophic)*	✗ High production cost ✗ Scale-up ✗ Novel food law ✗ Bioaccessability *(biomass)* ✗ Oxidative stability *(heterotrophic)*
Transgenic plants	✓ New supply ✓ Well-established large scale infrastructure	✗ Public perception ✗ Regulation ? Oxidative stability?

1.4. DIETARY INTAKE

Having discussed both traditional and novel n-3 LC-PUFA sources, of which a schematic overview is given in Figure 1.3, it is interesting to verify to what extent they can satisfy the human needs for a dietary intake associated with the beneficial health effects that were discussed in 1.1.2. Several organizations worldwide (both global and national) have set up n-3 LC-PUFA intake recommendations based on the available health benefit research. With recommended daily intakes varying from 100 to 1.600 mg EPA+DHA [32], these cover a broad range of values. However, the most commonly recommended intake amounts to 250-500 mg EPA+DHA/day. For pregnant or lactating women or people suffering from CVD, usually higher amounts are recommended [32,127]. Several organizations also recommend a fish consumption of 2 servings per week rather than a daily n-3 LC-PUFA intake. Given the decreasing n-3 LC-PUFA content of farmed fish, these recommendations may however need to be revised [38].

With a mean global consumption of 163 mg EPA+DHA/day the recommended intake is generally not reached [10]. Although regions characterized by a high seafood consumption such as Southeast Asia do reach beneficial intake values (710 mg EPA+DHA/day), the daily n-3 LC-PUFA intake of the major part of the global population (81,1%) is lower than the recommended value of 250 mg. Approximately two-thirds of the global population (66,8%) does not even acquire a daily intake of 100 mg [10]. Fish, the most traditional and most important n-3 LC-PUFA source covering almost 90% of our current n-3 LC-PUFA supply, therefore does not seem to suffice in providing a global, adequate n-3 LC-PUFA intake [4]. Although a low fish consumption and therefore a low n-3 LC-PUFA intake can partially be caused by a high cost or a dislike of flavor [128], the existing, finite fish stock is simply too small to meet the global demand of n-3 LC-PUFA. Considering an individual intake of 500 mg EPA+DHA/day, the annual global gap between n-3 LC-PUFA demand and supply has been estimated on 1.1 million tons, with fish only contributing to 15% of the global demand [107]. This emphasizes the importance of resorting to novel sources to support a healthy n-3 LC-PUFA intake. Of these novel sources, krill (as most important alternative marine source) currently contributes to only 0,3% of the demand, and would only allow a sustainable increase up to 8-9% [129]. It is therefore clear that either microalgae or transgenic plants (and accordingly microalgal genes) will need to be addressed for reassuring an adequate n-3 LC-PUFA supply.

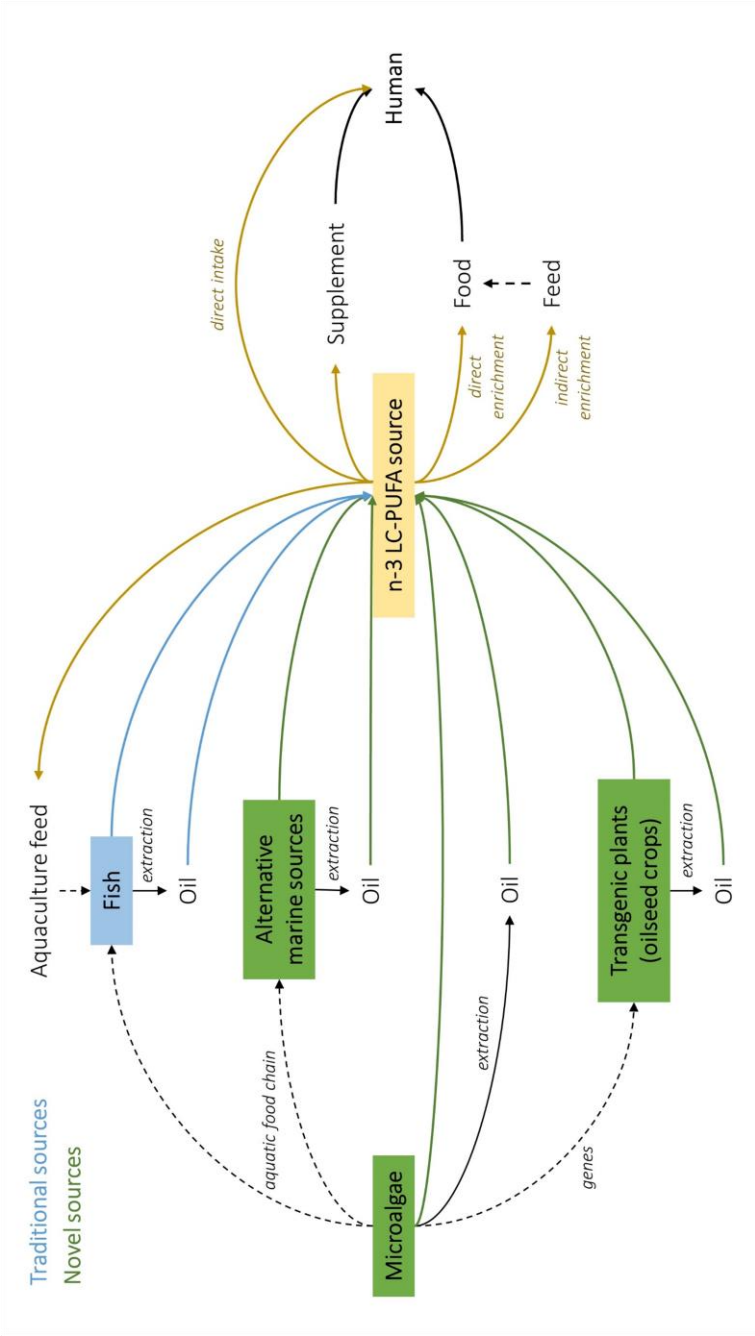


Figure 1.3: Traditional and novel food sources and their dietary intake (partially based on Kitessa et al. [114])

Finally, it is important to remark that even if sufficient n-3 LC-PUFA will become available in the future, they should still be consumed in a sufficient way to guarantee the beneficial health effects. As represented in Figure 1.3, this consumption is possible in several ways. Direct consumption is the most straightforward option, but increasing n-3 LC-PUFA intake by increasing direct consumption would require a change in eating habits that is not always straightforward [12]. Additionally, the n-3 LC-PUFA sources (especially the extracted oils) can be implemented in food supplements, although these have been shown to only reach a small part of the population [130]. Furthermore, n-3 LC-PUFA sources can be used to enrich commonly consumed food products. As this does not require a major change in diet, this is considered an innovative way of increasing n-3 LC-PUFA intake [131]. Apart from implementing n-3 LC-PUFA sources in food products in a direct way, they can also be added to the feed of farm animals and thereby increase the n-3 LC-PUFA content of their eggs, meat or milk in an indirect way [132–134]. The enrichment of food products again raises several technological challenges (for example in terms of oxidation), for which an aimed choice (for example based on oxidative stability) of n-3 LC-PUFA rich source will be required [131]. Lipid oxidation of n-3 LC-PUFA is the subject of the next literature review chapter.

CHAPTER 2 - OXIDATION OF LONG-CHAIN OMEGA-3 POLYUNSATURATED FATTY ACIDS

When studying n-3 LC-PUFA, lipid oxidation is of major concern due to the high susceptibility of these fatty acids towards this undesired chemical deterioration process. In this chapter the general aspects of lipid oxidation (2.1) and, its counterpart, antioxidation (2.2) are discussed in terms of definition, mechanism, affecting factors and analysis. In the second section also the most important antioxidants (with a major focus on microalgal ones) are being touched upon. Against the background of this general knowledge, former research onto how oxidation and antioxidation determine the oxidative stability of n-3 LC-PUFA rich systems in specific is discussed (2.3). Regarding the different n-3 LC-PUFA sources that were discussed in Chapter 1, microalgae are from now on mainly focused upon because of their central role as research subject in the current PhD research.

2.1. OXIDATION

2.1.1. DEFINITION

Lipid oxidation is the process in which lipids chemically degrade upon reaction with oxygen. According to Farhoosh [135] it is the most primary event affecting their stability and safety in both foods and living systems. Regarding food products, the degradation is undesired as it troubles the quality in three different ways: it produces a rancid off-flavor due to the formation of volatile compounds, it endangers the products' safety as oxidation products with potentially toxic effects could be formed, and in case of lipid compounds with health beneficial properties like n-3 LC-PUFA it results in a loss of nutritional value due to their breakdown [15].

2.1.2. MECHANISM

The general mechanism of lipid oxidation is two-phasic. In a first step, denoted as the primary oxidation, lipids (more specifically the fatty acid moieties of the different lipid species) are converted into lipid hydroperoxides as primary oxidation products. Subsequent secondary oxidation consists of the further reaction of these hydroperoxides into a broad range of secondary oxidation products among which aldehydes, ketones, alcohols, epoxides, dimers and polymers [7]. Depending on the exact pathways or circumstances under which the lipids react, three types of lipid oxidation can be distinguished: autoxidation, photo-oxidation and enzymatic oxidation. However, they all three follow the general sequence of primary oxidation towards hydroperoxides and secondary oxidation from hydroperoxide decomposition [136]. The mechanism of both autoxidation and photo-oxidation will be discussed in more detail below.

Enzymatic oxidation, which is oxidation catalyzed by the action of certain enzymes like lipoxygenases, is not further considered since former research has shown it to be of minor relevance for the systems that were studied in the current PhD research [9].

2.1.2.1. AUTOXIDATION

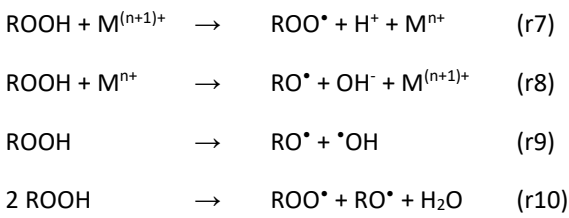
Autoxidation is the type of lipid oxidation occurring spontaneously by an autocatalytic radical chain mechanism. It can be described as a sequence of initiation, propagation and termination according to the following reactions:

Initiation:	RH	→	$R^{\bullet} + {}^{\bullet}H$	(r1)
Propagation:	$R^{\bullet} + O_2$	→	$ROO^{\bullet}$	(r2)
	$ROO^{\bullet} + RH$	→	$ROOH + R^{\bullet}$	(r3)
Termination:	$ROO^{\bullet} + ROO^{\bullet}$	→	$ROOR + O_2$	(r4)
	$ROO^{\bullet} + R^{\bullet}$	→	ROOR	(r5)
	$R^{\bullet} + R^{\bullet}$	→	RR	(r6)

During the initiation phase a lipid radical $R^{\bullet}$ is being formed (r1). The hydrogen abstraction necessary to do so is induced by another radical (initiator) formed under the influence of heat, metals or UV light. The subsequent propagation phase consists of a fast reaction of this lipid radical with (triplet) oxygen to form a lipid peroxyl radical $ROO^{\bullet}$ (r2), and a slower, rate determining hydrogen abstraction by this peroxyl radical from another lipid resulting in the formation of a lipid hydroperoxide ROOH, the primary oxidation product, and a new lipid radical (r3). This lipid radical can again go through these propagation reactions, explaining the autocatalytic aspect. Termination occurs when radicals react with each other (r4-r6) to form non-radical products that no longer propagate the radical chain [7].

Although the above process of forming lipid hydroperoxides suggests a straightforward sequential mechanism of oxidation, more complexity is involved due to the appearance of alternate reactions. These comprise rearrangement, addition or scission reactions of which radical reaction products can either further propagate the radical chain process and result in the formation of hydroperoxides (primary oxidation) or undergo further reaction towards a broad range of so-called 'secondary oxidation products' (secondary oxidation) [137]. Moreover, the

scission reactions, which are also called branching reactions, can occur in different ways:



$$ROOH + M^{(n+1)+} \rightarrow ROO^{\bullet} + H^{+} + M^{n+} \quad (r7)$$

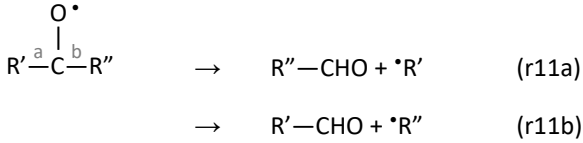
$$ROOH + M^{n+} \rightarrow RO^{\bullet} + OH^{-} + M^{(n+1)+} \quad (r8)$$

$$ROOH \rightarrow RO^{\bullet} + {}^{\bullet}OH \quad (r9)$$

$$2\ ROOH \rightarrow ROO^{\bullet} + RO^{\bullet} + H_2O \quad (r10)$$

Reactions (r7) and (r8) show a heterolytic scission under the influence of metal ions, while reactions (r9) and (r10) are homolytic and can respectively occur monomolecularly or bimolecularly. Especially the latter has been reported to be a dominant reaction in the propagation phase of autoxidation [135,138].

Regarding secondary oxidation a big diversity of compounds can be obtained. Internal rearrangement and addition reactions result in the formation of non-volatile products (respectively epoxides and dimers or polymers), whereas volatile compounds (mainly responsible for the undesirable flavor formation) usually originate from the scission reactions [137]. A homolytic β-scission of one of the two carbon-carbon bonds (a or b) of alkoxy radicals $RO^{\bullet}$, resulting from scission of hydroperoxides, is considered as a main pathway for the formation of short-chain volatile compounds:



$$R'\overset{a}{-}\overset{\overset{\displaystyle O^{\bullet}}{|}}{C}\overset{b}{-}R'' \rightarrow R''-CHO + {}^{\bullet}R' \quad (r11a)$$

$$\rightarrow R'-CHO + {}^{\bullet}R'' \quad (r11b)$$

Additionally, if unsaturated, these secondary oxidation products can still further oxidize [7,139].

2.1.2.2. Photo-oxidation

Photo-oxidation is oxidation occurring under the influence of light, which covers both UV and visible light. The incidence of UV light can result in the formation of free radicals which can initiate the autoxidation mechanism such as discussed in 2.1.2.1. For visible light to induce lipid oxidation, the presence of a so-called photosensitizer is required. When exposed to light of a specific wavelength the ground singlet state of a photosensitizer (^{1}Sen) becomes excited ($^{1}Sen^{*}$) and can be converted to an excited triplet state ($^{3}Sen^{*}$) (r12). By returning to its ground

state, this excited triplet photosensitizer can provoke photo-oxidation in two different ways. Type I photosensitizers (like riboflavin) cause the formation of a free radical (r13) which can again initiate the autocatalytic radical chain mechanism (type I photo-oxidation). Type II photosensitizers (like chlorophyll) can bring oxygen ($^3O_2$) to a highly reactive singlet state ($^1O_2$) (r14), which can, due to its high electrophilicity, subsequently oxidize unsaturated lipids into hydroperoxides via direct addition (r15) (type II photo-oxidation) [140].

Excitation:	^{1}Sen	$\xrightarrow{h\nu}$	^{1}Sen* → ^{3}Sen*	(r12)
Type I:	^{3}Sen* + RH	→	^{1}Sen + R$^\bullet$ + $^\bullet$H	(r13)
Type II:	^{3}Sen* + $^3O_2$	→	^{1}Sen + $^1O_2$	(r14)
	$^1O_2$ + RH	→	ROOH	(r15)

Whereas photo-oxidation promoted by UV light and type I photo-oxidation leads to similar hydroperoxide species (i.e. with the hydroperoxide moiety on similar carbon atoms within the fatty acid chain) as for autoxidation, the hydroperoxide distribution is different for type II photo-oxidation due to this different mechanism [7]. Just like for autoxidation, the further decomposition of formed hydroperoxides leads to a diversity of secondary oxidation products, which may also be altered in the case of type II photo-oxidation since another hydroperoxide distribution is started from [141].

2.1.3. FACTORS AFFECTING OXIDATION

The appearance and degree of lipid oxidation is affected by several factors which are related to either substrate, environment or system. The most important ones are discussed below.

2.1.3.1. SUBSTRATE PROPERTIES

Since lipids are the substrate to be oxidized, their intrinsic properties highly influence the lipid oxidation rate.

Firstly, the fatty acid composition is of importance: a higher degree of unsaturation is generally associated with a higher lipid oxidation susceptibility. However, rather than the amount of double bonds, the amount of allylic and (especially) bisallylic hydrogen atoms determines the autoxidation rate. These are hydrogen atoms bound to a carbon atom adjacent to respectively one or two double bonds (Figure 2.1). This can be explained by the lower bond dissociation

energy of (bis)allylic hydrogens that has to be overcome in the rate limiting propagation step (r3): the formed radical can become distributed across a resonance stabilized double bond system [142]. For type II photo-oxidation on the other hand, the number of double bonds is determinative due to the direct addition mechanism discussed in 2.1.2.2. [143]. With n-3 LC-PUFA having both a high amount of double bonds and bisallylic hydrogen atoms, their high susceptibility towards lipid oxidation is explained.

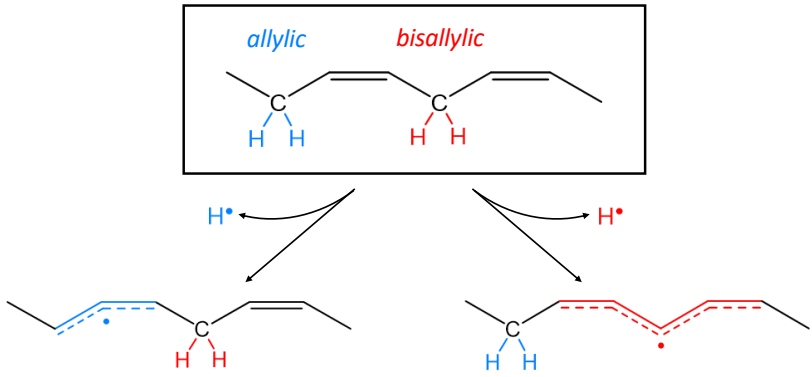


Figure 2.1: Allylic and bisallylic hydrogen abstraction

Secondly, also the impact of lipid class has been described, albeit less unambiguous. PLs and free fatty acids (FFAs) show other oxidation characteristics as compared to TAGs, and although they are presumed to respectively inhibit and promote lipid oxidation, the opposite has been reported for both lipid classes as well [143,144]. For glycolipids (GLs), a higher oxidative stability in comparison with TAGs has been described [145].

Finally, also the position of the fatty acids within a certain lipid seems to matter since TAGs have been described as being more stable with unsaturation on the sn-2 position rather than on the sn-1 or sn-3 position [143,146].

2.1.3.2. Environmental properties

Environmental factors comprise conditions of oxygen, temperature and water. Light can also be seen as an influencing environmental factor, but has already been covered in the explicit section about photo-oxidation (2.1.2.2.).

For oxygen, since it directly takes part in the oxidation reaction, it should not surprise that its concentration in headspace and matrix (determined by solubility and diffusivity) has an effect on the lipid oxidation rate. However, this is only noticeable at low concentrations, where a decrease in concentration is associated with a slower oxidation rate [142].

Regarding temperature, an increase of 10 °C corresponds with a two-fold increase of the oxidation rate [147]. This should however not be extrapolated to temperatures higher than 60 °C since other oxidation mechanisms then occur due to a reduced oxygen solubility [139]. Sullivan & Budge [148] even suggested not to use this principle above 40 °C for high PUFA oils, since they observed other oxidation kinetics in that case. For type II photo-oxidation, temperature is only of low relevance due to the already rapidly occurring singlet oxygen addition [140].

The influence of water has been described in terms of water activity with both high and low values leading to an increase in lipid oxidation respectively caused by a higher mobility and accessibility [143].

2.1.3.3. SYSTEM PROPERTIES

An important aspect in considering lipid oxidation affecting factors is the system that is being considered. Not only does it affect the influence of both substrate properties (oxidation inhibiting or promoting effects of FFAs and PLs) and environmental properties (oxygen concentration and location) [143,144], it also provides additional factors. In the case of oil-in-water emulsions (O/W emulsions), systems that are often contrasted with bulk oils in general lipid oxidation literature [143,149], droplet size and emulsifier type and concentration become key parameters because of the interfacial area being the critical area where lipid oxidation occurs [150]. The influence of similar oxidation affecting factors (like the ones discussed in 2.1.3.1. and 2.1.3.2.) is therefore hard to compare in different systems.

2.1.4. ANALYSIS

2.1.4.1. GENERAL

For the analysis of lipid oxidation, numerous methods have been described, differing in simplicity, specificity and sensitivity. They either measure reagents, by determining the oxygen absorption or the loss of initial (lipid) substrate, or (more frequently) reaction products, being either primary (hydroperoxides) or secondary oxidation products [151,152]. For an extensive comparison of the different

methods the reader is referred to the reviews of Barriuso et al. [153] and Abeyrathne et al. [154].

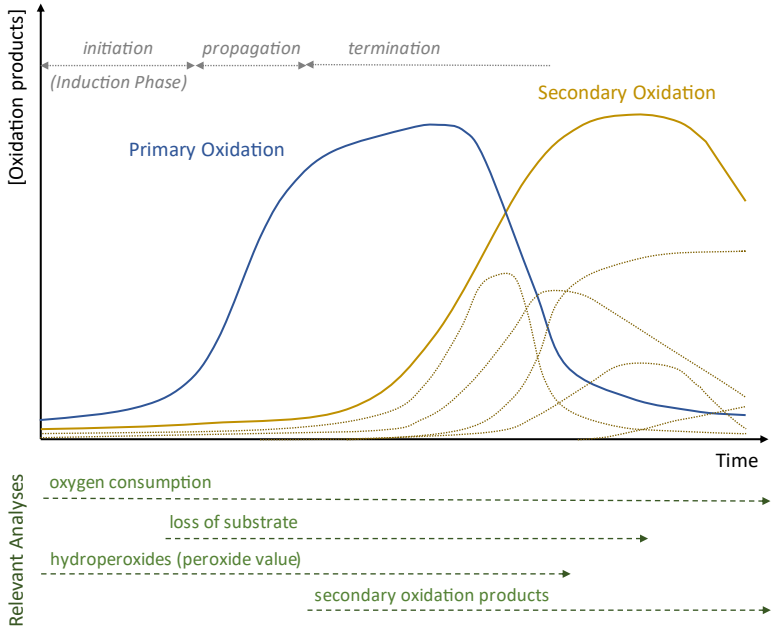


Figure 2.2: Evolution of lipid oxidation products and relevant analyses (based on Farhoosh [135], Frankel [7] and Schaich et al. [143]). The dotted curves are a representation of possible single secondary oxidation product evolutions, of which the solid 'secondary oxidation' curve is the sum. The represented curves are purely indicative in time as well as in order of magnitude.

Regardless of the analysis method of choice, when analyzing lipid oxidation, the evolution of oxidation throughout storage should be considered. Figure 2.2 shows the evolution of both primary and secondary oxidation products. The two-phasic process that was discussed as a general mechanism in 2.1.2. can be recognized by the appearance of a primary and a secondary oxidation peak. This sequence applies for both autoxidation and photo-oxidation, although for type II photo-oxidation the time period over which this occurs is expected to be shorter due to the fast nature of singlet oxygen addition [140]. Considering autoxidation and type I photo-oxidation (i.e. radical mediated oxidation) the flow of initiation, propagation and termination can be recognized in the primary oxidation peak by respectively a slow increase (also called the 'induction phase'), an exponential

increase, and a retardation followed by decrease. The latter can be explained by the rate of hydroperoxide decomposition (towards secondary oxidation products) becoming higher than the rate of hydroperoxide formation (which is decreasing by running out of lipid substrate or by termination reactions canceling out radicals that propagate the radical chain progress). While the primary oxidation peak only consists of lipid hydroperoxides as primary oxidation products, the secondary oxidation peak represents the sum of the large variety of possible secondary oxidation products. For this reason, the possible evolution of underlying single secondary oxidation products was added to Figure 2.2. Specific secondary reaction products can be formed in an early or late phase, and can further decompose (e.g. a further oxidation in the case of remaining unsaturations) or stay present. The represented curves are purely indicative in time as well as in order of magnitude [7,135,143].

Apart from the evolution of primary and secondary oxidation, Figure 2.2 also shows the relevant time frame of the different types of analyses. Only oxygen absorption analyses remain relevant throughout the whole storage time since for both primary and secondary oxidation oxygen can be consumed [143]. Analyses measuring the loss of initial substrate were not indicated as being relevant from the beginning due to their lower sensitivity as compared to measuring primary oxidation products [152]. The evolution of lipid oxidation illustrates two general points of attention regarding its analysis: multiple analyses should be performed to be informative towards the state of lipid oxidation [137,153], and analyses should be done over multiple time points [135]. These were applied in the current PhD research by measuring primary oxidation products, a range of specific secondary oxidation products and the loss of n-3 LC-PUFA, as most susceptible initial substrate, throughout storage time. The analyses used for analyzing primary and secondary oxidation products are further focused upon in section 2.1.4.2.

Storage experiments are often performed at temperatures higher than room temperature to accelerate the oxidation process. This reduces the required analysis time frame. As was discussed in section 2.1.3.2., at higher temperatures other oxidation mechanism may occur. Therefore, the choice of a suitable storage temperature is important for the results to be representative for oxidation processes at room temperature. Accelerated methods like Rancimat, going to elevated temperatures above 100 °C [155], should therefore not be used.

2.1.4.2. PRIMARY OXIDATION

Traditionally the amount of hydroperoxides, expressed as the PV is determined by titration (standard method) or spectrophotometry. However, more complex methods with increased specificity and sensitivity have arised using (among others) nuclear magnetic resonance, infrared spectrometry and chromatography [153]. Although when studying microalgae, the presence of pigments troubles the use of spectrophotometric methods, Gheysen et al. [156] developed a simple, low-cost spectrophotometric method (adapted ferrous oxidation xylenol orange method) that overcomes this problem and has proven to be suitable for the determination of primary oxidation in systems rich in microalgae or microalgal substances. Therefore, this method is used throughout the current PhD research.

2.1.4.3. SECONDARY OXIDATION

Whereas primary oxidation analyses usually only measure the total amount of lipid hydroperoxides, secondary oxidation analysis can be focused on several analytes due to the diversity of secondary oxidation compounds. This opens up opportunities to measure several oxidation markers with high specificity [139,143].

The type and amount of volatile compounds depend on the fatty acid composition of the oxidizing lipids [139]. Following the evolution of a certain volatile throughout storage does therefore not only tell something about the degree of oxidation, but also about the identity of the oxidizing fatty acids [152]. A frequently reported distinction is the one between hexanal and propanal respectively originating from the oxidation of n-6 and n-3 fatty acids [153]. When investigating the oxidation of n-3 LC-PUFA rich lipids (like in the current PhD research), compounds specifically originating from their oxidation are therefore of major interest. Looking for this information in former research mainly led to studies on the oxidation of fish or fish oil (rich) products in which the rancid flavor formation and its association with the development of certain volatile compounds is generally of higher relevance than the mechanistic relation with oxidizing n-3 LC-PUFA [7,157–159]. Although in these studies an increase of a multitude of volatile compounds upon oxidation could be detected, this gives no certainty about the specificity of these compounds for n-3 LC-PUFA oxidation. Limited mechanistic information on certain volatile compounds from n-3 LC-PUFA oxidation can however be found in generally older research papers. The compounds in question are the following and were given priority when considering secondary oxidation in the current PhD research: 2-pentenal, 2,4-heptadienal, 3,5-octadien-2-one, 2,6-nonadienal,

3,6-nonadienal and 2,4,7-decatrienal [160–163]. Figures 2.3 and 2.4 show their formation mechanism starting from EPA. The formation mechanism starting from DHA is similar. Additionally, 2-butenal is being reported as a compound originating from singlet oxygen oxidation of n-3 fatty acids [161]. A direct addition of singlet oxygen can cause the formation of a specific hydroperoxide that is not likely to be formed by autoxidation (17-OOH for EPA, 19-OOH for DHA). 2-Butenal results from a subsequent β-scission and could therefore serve as a marker for secondary type II photo-oxidation. However its possible formation from decadienal has also been described [164]. Although no reaction pathway was found for the first, 2,4-decadienal has been described as a typical oxidation product of both n-3 and n-6 fatty acids.

For measuring volatile compounds, gas chromatography (GC) is a suitable technique. Different sampling methods can be applied, with increasing complexity: static headspace, solid phase microextraction (SPME) and dynamic headspace. Each one presents its own advantages and shortcomings [153]. For SPME, volatiles are being preconcentrated on a fiber (adsorption) and subsequently released into the GC injection port by thermal desorption. It is the most widely used sampling method [165] and it is stated to be as sensitive but less expensive as compared to a dynamic headspace set-up [166,167]. Recently an improved SPME technology has emerged: SPME Arrow [168]. It offers a higher sample capacity due to a larger sorbent phase volume and the fiber has a higher robustness, making the analysis more durable [169]. Also, specifically for triple phase divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers, it resolves the multiphase irregularities encountered by conventional SPME, leading to a better interdevice reproducibility [170]. Former research with SPME Arrow on food matrices is limited to volatile compound analysis in soy sauce [168], brown rice vinegar [169], fermented fish sauce [169] and milk samples [171], but all of these studies reported an increase in both sensitivity and reproducibility as compared to conventional SPME. Compounds were not only reported to be analyzed in 'higher levels', the number of different compounds was also higher indicating a broader volatile compound analysis [165].

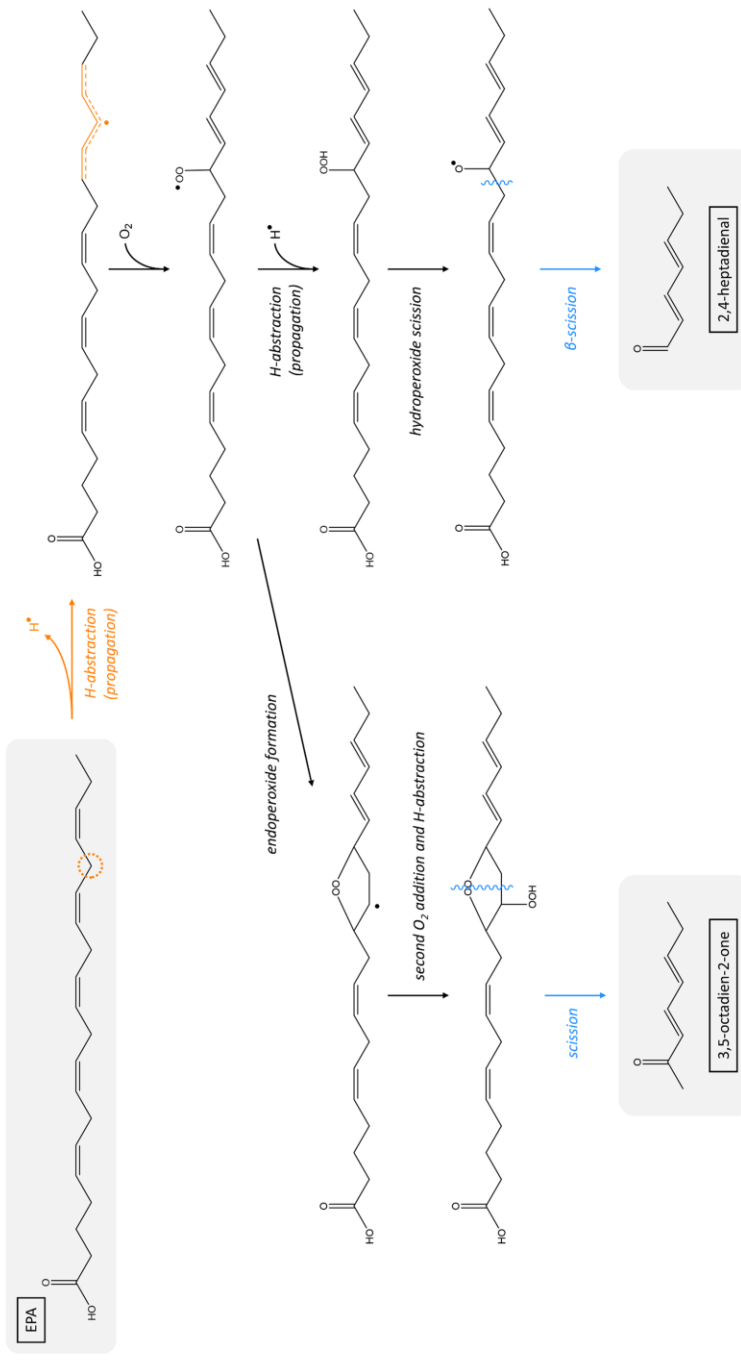


Figure 2.3: 2,4-heptadienal and 3,5-octadien-2-one formation mechanism from EPA (based on Noble & Nawar [162] and Lee et al. [160])

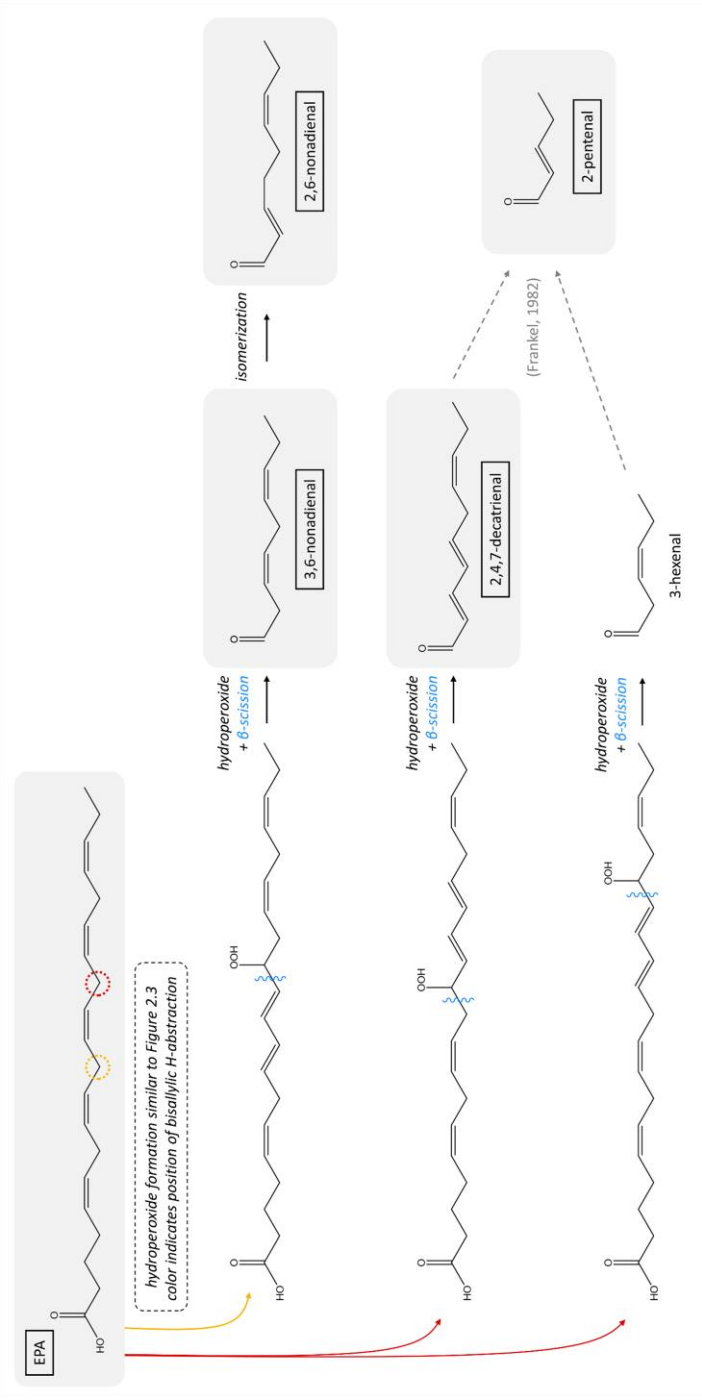


Figure 2.4: 3,6-nonadienal, 2,6-nonadienal, 2,4,7-decatrienal and 2-pentenal formation mechanism from EPA (based on Frankel [7,161] and Noble & Nawar [163])

2.2. ANTIOXIDATION

2.2.1. DEFINITION

Antioxidation is the process of chemically delaying or inhibiting oxidation. It is accomplished by so-called antioxidants which are defined as 'compounds that retard or prevent the oxidation of an oxidizable substrate while being present in low concentrations in comparison to that of the oxidizable substrate' [149]. Although other, physical measures might help in the prevention of oxidation (lowering temperature, excluding light etc.), antioxidants are considered as the most important defense mechanism [15]. Therefore, from a food product point of view, they play a critical role as food preservative in the food industry [172]. Also from a biological (human health) point of view they could be advantageous due to their involvement in the prevention of oxidative stress and related diseases [173], although this aspect is not further discussed upon in this PhD.

2.2.2. MECHANISM

Although antioxidants comprise a broad range of compounds (as will become clear in section 2.2.5.), they can broadly be divided into three different classes depending on their working mechanism: preventive, chain-breaking and quenching.

Preventive antioxidants act on the initiation phase of autoxidation. By either decomposing (hydro)peroxides or chelating metal ions, they inhibit the formation of radicals that could serve as possible initiators and therefore decrease the rate of initiation [149].

Chain-breaking or peroxyl radical trapping antioxidants are of importance in the propagation phase. They limit its rate by directly reacting with the peroxyl radicals according to reaction (r16).

$$AH + ROO^{\bullet} \rightarrow A^{\bullet} + ROOH \qquad (r16)$$

The antioxidative property relies on the antioxidant radical $A^{\bullet}$ having a higher stability than the peroxyl radical (generally due to possible resonance delocalization) and therefore not being inclined to abstract a hydrogen from another lipid molecule. In this way propagation is avoided [174]. Moreover, the formed antioxidant radical can result in termination reactions (r17-18), possibly trapping another peroxyl radical [7] as shown in reaction (r17).

$A^{\bullet} + ROO^{\bullet} \rightarrow ROOA$ (r17)

$A^{\bullet} + A^{\bullet} \rightarrow AA$ (r18)

Reaction (r16) can occur via several mechanisms: hydrogen atom transfer, proton-coupled electron transfer, electron transfer-proton transfer and sequential proton-loss electron transfer. Suppression of propagation lasts until the chain-breaking antioxidants are fully consumed [149].

Whereas preventive and chain-breaking antioxidants limit autoxidation, **quenching antioxidants** specifically inhibit photo-oxidation. This quenching concerns the deactivation of the excited state of either photosensitizers or singlet oxygen to their ground state, and can occur either physically or chemically. Physical quenching involves deactivation without oxygen consumption or product formation. Chemical quenching on the other hand involves oxidation of the antioxidant that is therefore consumed [174].

Finally, an important effect to point out when considering the mechanism of antioxidation is the occurrence of **synergism**: mixtures of antioxidants can have a stronger antioxidative effect than the single compounds. This not only by the combination of the above discussed classes acting on different lipid oxidation sites, but also by the appearance of so-called co-antioxidants which are able to regenerate a certain antioxidant. A classic example of this is the regeneration of tocopherol by ascorbic acid. Carotenoids can in their turn be regenerated by tocopherols or ascorbic acid [172,175].

2.2.3. FACTORS AFFECTING ANTIOXIDATION

Although influencing factors regarding antioxidation are very much dependent on the antioxidants' compound class (see 2.2.5.), just like for lipid oxidation, some general aspects regarding substrate, environmental and system properties can be recognized.

2.2.3.1. SUBSTRATE PROPERTIES

It is self-evident that the antioxidants' structure determines its activity, for whatever compound. Dependent on the above discussed working mechanisms, the influencing substrate properties vary: the effectiveness of a chain-breaking antioxidant is dependent on its resonance stabilization and therefore the range of its conjugated system and the presence of functional groups [176], while for a quenching antioxidant mainly the number of conjugated double bonds

(determining the lowest triplet energy level) matters [177]. Furthermore, structure determines mobility which is important for the ease with which interaction with lipid oxidation occurs [149]. Finally, substrate properties like polarity have important implications for the localization within the system (considered in 2.2.3.3.), which can be key in order to obtain antioxidative effects.

2.2.3.2. ENVIRONMENTAL PROPERTIES

Regarding oxygen and temperature, some specific effects for antioxidation can also be mentioned. Certain compounds only act as an antioxidant under low partial pressure of oxygen. When oxygen pressure is too high, antioxidant peroxyl radicals can be formed which could, on the contrary, propagate the lipid oxidation process [177,178]. Also temperature can affect antioxidant activity: upon comparing series of antioxidants, and making abstraction of the fact that lipid oxidation mechanisms additionally change upon high temperature increases, inversion of activity can occur with increasing temperature. This makes it also for antioxidation, hard to extrapolate findings between higher (>100 °C) and lower (<60 °C) temperatures [149].

2.2.3.3. SYSTEM PROPERTIES

Antioxidants do not always act in the same way: for antioxidation as well as lipid oxidation a lot is determined by the system under consideration and a distinction between bulk oils and O/W emulsions is often made. The polar paradox is generally referred to. It states that polar antioxidants are more effective in bulk oils (although it concerns a nonpolar matrix) while nonpolar antioxidants have a higher activity in O/W emulsions (a system with a polar matrix). Both phenomena are being addressed to the fact that antioxidants of opposite polarity can be found in higher concentrations at the site of oxidation: the air-oil interface for bulk oils, the oil-water-interface for O/W emulsions. However more complexity is involved. For bulk oils, association colloids instead of the air-oil interface would be the major site of oxidation which causes additional factors like the amount of trace water, transition metals and PLs to be of importance. For O/W emulsions cut-off effects appear in which a too low polarity is disadvantageous. To date, no general rule or theorem can be applied to predict the efficiency of a certain antioxidant in a generic system due to the complexity and interplay of influencing factors. Therefore, antioxidative action should be approached in a more specific way with a high caution to extrapolating findings across different systems [179].

2.2.4. ANALYSIS

Similarly to the multitude of methods for analyzing lipid oxidation, various methods are available to analyze antioxidation or, in other words, the capacity of certain antioxidants. A distinction should be made between doing this in a direct way, or in an indirect way (which comes down to assessing antioxidative effects by measuring an effect on lipid oxidation as discussed in 2.1.4).

2.2.4.1. DIRECT

Direct analysis covers a broad range of in vitro assays to assess the antioxidant capacity of single compounds using model systems. An extensive overview of the different methods covering their working principles and (dis)advantages can be found in Abeyrathne et al. [154]. Although these assays are simple and inexpensive they have several drawbacks. First of all a single measurement cannot reflect the complex kinetics of lipid oxidation throughout storage [149]. Secondly, they assess only one antioxidative working mechanism, while certain antioxidants can act via multiple mechanisms depending on the working conditions. This necessitates the use of multiple assays. Thirdly, they often use artificial probes rather than biologically relevant reactive species making it hard to extrapolate findings to real systems [180]. Although these assays can give an indication on the activity order of a certain antioxidant series, the results are often 'misleading' to generalize due to these drawbacks and should therefore be handled with care [149].

2.2.4.2. INDIRECT

Indirect analysis estimates the effectiveness of antioxidants by evaluating the appearance and extent of lipid oxidation throughout storage. The stability of an inhibited (antioxidant present) and an uninhibited (antioxidant absent) system is compared. Although this does not lead to detailed knowledge on the antioxidation mechanism of a certain compound [149], it gives sufficient information to evaluate the practical effect in a real system [180]. However, it goes along with a higher labor-intensity than for direct methods, since oxidation products have to be evaluated throughout storage.

2.2.5. IMPORTANT ANTIOXIDANTS WITH A MICROALGAL FOCUS

2.2.5.1. NATURAL VS. SYNTHETIC

Considering important antioxidants, a distinction can be made between synthetic and natural antioxidants. Although synthetic antioxidants – of which BHA, BHT,

tert-butylhydroquinone (TBHQ) and propyl gallate (PG) are important representatives - possess strong antioxidative capacities and are most widely used to inhibit oxidation in food products [15], natural antioxidants keep on gaining interest. This is because of the use limiting toxicological safety concerns (e.g. causing DNA damage) and a diminished consumer acceptability of the synthetic ones [172,181].

Although plant sources are frequently addressed in the search for natural antioxidants, photoautotrophic microalgae are a rich source as well. The presence of antioxidants is a direct consequence of the microalgae's lifestyle. They serve as a natural defense system to counteract the harmful effects of reactive oxygen species (ROS) being generated as by-products upon physiological processes in chloroplasts, mitochondria and peroxisomes [182]. Among these antioxidants, carotenoids are the major point of focus of the current PhD research, with their antioxidative properties being discussed in the next section (2.2.5.2.). Other antioxidative compounds present in microalgae are considered thereafter (albeit in a less extensive way) (2.2.5.3.).

2.2.5.2. CAROTENOIDS

Carotenoids are a diverse group of tetraterpenoid pigments absorbing light at wavelengths mainly between 400 and 500 nm. Their structure, made up of 40 carbon atoms (eight isoprene units), varies in number of conjugated double bonds, the appearance of cyclic end-groups and their substitution with oxygen-containing functional groups. This results in the emergence of over 700 different carotenoids, which can be classified in carotenes (containing no oxygen) and xanthophylls (containing at least one oxygen) [183]. Figure 2.5 shows the structure of several important microalgal carotenoids. Despite the wide diversity, with some characteristic carotenoid profiles being able to serve as taxonomic markers, no more than 30 different carotenoids carry out a direct role in the microalgae's photosynthesis [184]. This physiological role is twofold: on the one hand they are important for supplemental light absorption (from wavelengths that cannot be captured by chlorophyll), on the other hand they protect the photosynthetic system against oxidative damage underlining their antioxidative properties.

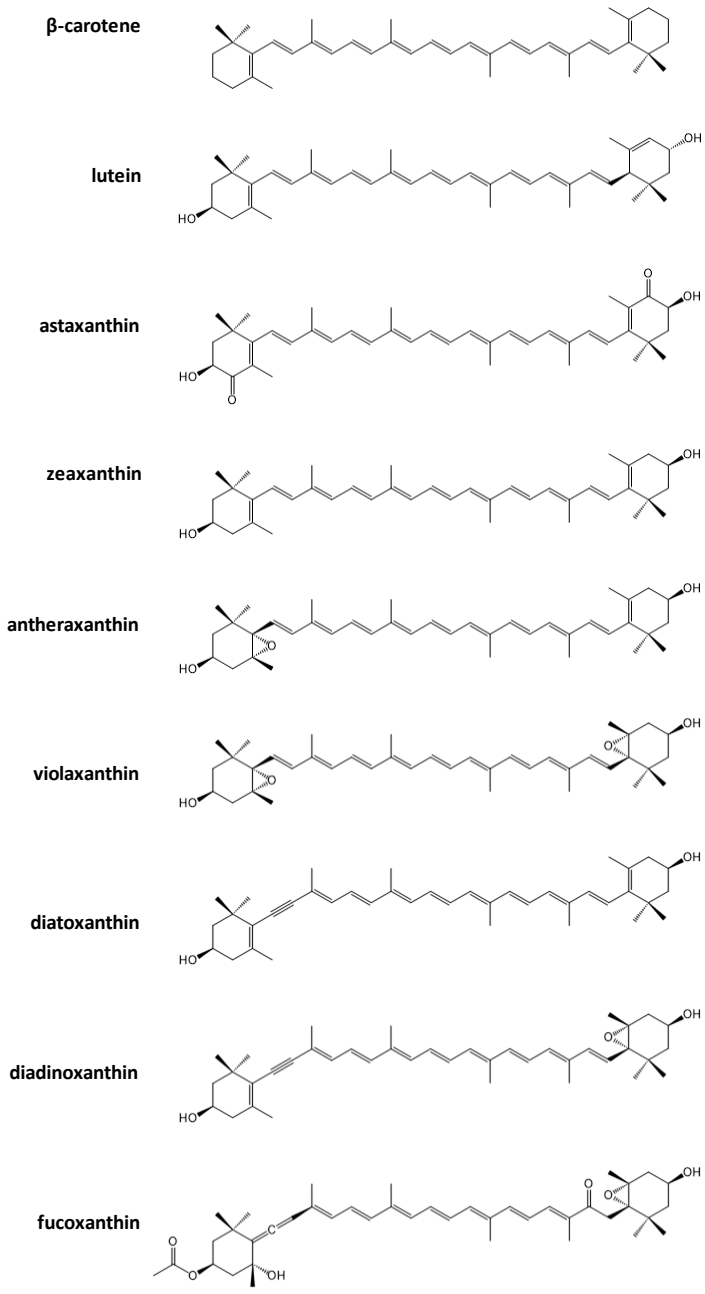


Figure 2.5: Chemical structures of several important microalgal carotenoids

Carotenoids contain both quenching and chain-breaking (free radical scavenging) properties. Regarding the quenching part, both singlet oxygen and excited chlorophyll (^{1}Chl*) can be deactivated upon formation of a triplet excited carotenoid, subsequently returning to its ground state by heat dissipation (physical quenching). Although chemical quenching can also occur, it is reported to be of minor importance as it would represent less than 0.05% of the quenching [175]. As opposed to chemical quenching the carotenoid structure remains intact upon physical quenching. However, after several quenching cycles (reported up to 1000 for β-carotene), the carotenoid is eventually oxidized [173,185]. For the scavenging of peroxyl radicals three different mechanisms can occur: electron transfer (r19), hydrogen abstraction (r20) and addition (r21).

$$Car + ROO^{\bullet} \rightarrow Car^{\bullet+} + ROO^{-} \quad (r19)$$

$$Car + ROO^{\bullet} \rightarrow Car(-H)^{\bullet} + ROOH \quad (r20)$$

$$Car + ROO^{\bullet} \rightarrow ROO\text{-}Car^{\bullet} \quad (r21)$$

The mechanism taking place largely depends on the polarity of the environment, with electron transfer being favored in more polar environments and hydrogen abstraction and addition in non-polar ones [186]. Either way, a propagating peroxyl radical is canceled out and a more stabilized radical is obtained, accompanied by a loss of the original carotenoid structure.

A high diversity in structure implies differences in antioxidative capacities. The double bond pattern (number, conjugation and cis-trans-configuration) and the presence of functional groups (oxo, hydroxyl, epoxide) have been reported as influencing factors in this regard [15,175,177,187–189]. A clear ranking in antioxidative efficiency based on structure is however not self-evident. Not only did Müller et al. [190] observe different orders depending on the analysis that was used (albeit all direct analyses), also (as was discussed in 2.2.3.) the environmental and system properties play an important role. For carotenoids, regarding environmental properties, the oxygen pressure seems to be of importance: when this is too high, the radical formed in reaction (r21) has the tendency to form a new peroxyl radical upon reaction with oxygen (ROO-Car-OO$^{\bullet}$) which can again propagate the free radical chain process (by hydrogen abstraction) rather than inhibiting it [177,178]. Regarding system properties, the carotenoids' polarity seems to be decisive as it determines their localization. Xanthophylls would for example be more potent antioxidants in non-polar media [186]. Finally, also synergistic effects occur with both interactions with other carotenoids (mixtures more effective) and other

antioxidants (tocopherols and ascorbic acid) being described [172,173,175] (as was already touched upon in 2.2.2.).

2.2.5.3. OTHER NATURAL MICROALGAL ANTIOXIDANTS

Other natural antioxidants that are present in microalgae are antioxidative vitamins, phenolic compounds, glutathione and some species specific components.

Ascorbic acid (vitamin C) is a water-soluble antioxidative vitamin. It mainly serves as a preventive antioxidant since it can maintain the reduced state of metal ions and it can scavenge several ROS (superoxide, hydroxyl radicals and hydroperoxides) which could otherwise both serve as initiators of the lipid oxidation process [15,182]. Additionally it can act as a co-antioxidant regenerating both carotenoids and tocopherols [175,177].

Tocopherols (vitamin E) are lipophilic antioxidative vitamins. They have both chain-breaking and quenching properties since they can scavenge lipid peroxyl radicals and quench singlet oxygen (both physically and chemically) [15,177]. Both regeneration 'by' and regeneration 'of' carotenoids have been described [172].

Although mentioned separately, tocopherols also belong to the broader class of **phenolic compounds**. This is a large family of structures containing at least one aromatic ring with one or more hydroxyl groups. For microalgae simple phenolics, phenolic acids and flavonoids are relevant [177,191]. Next to their compositional variety, the phenolic antioxidative mechanism is diverse as well: they can act preventive (metal chelation and inhibition of pro-oxidative enzymes), chain-breaking (peroxyl radical scavenging) and quenching (singlet oxygen scavenging) [192].

Glutathione is a water-soluble tripeptide that can be found as an antioxidant in both animals and photosynthetic organisms. Although its antioxidative activity is most relevant from a physiological point of view (regeneration of ascorbate in the ascorbate-glutathione cycle and hydrogen peroxide decomposition with glutathione peroxidase), it can also scavenge several ROS (superoxide, hydroxyl radical and singlet oxygen) making it worth mentioning [177].

Finally, for the sake of completeness, the antioxidative properties of certain **species specific compounds** have also been reported. It concerns mycosporin-like amino acids, sulfated polysaccharides and phycobiliproteins [177,182].

2.3. OXIDATION AND ANTIOXIDATION DETERMINING THE OXIDATIVE STABILITY OF N-3 LC-PUFA

The oxidative stability of n-3 LC-PUFA is eventually determined by the interplay between a high oxidation tendency, whether or not reinforced by the above discussed adverse affecting factors, and the presence of a sufficient or insufficient amount of effective antioxidants. Photo-oxidation, on top of autoxidation, is one of the points of interest of the current PhD research (Chapter 4). Therefore, in considering oxidative stability differences of different n-3 LC-PUFA rich systems, a distinction is made between the most important findings of former research on autoxidative stability (2.3.1.) and photo-oxidative stability (2.3.2.).

2.3.1. FORMER RESEARCH ON AUTOXIDATIVE STABILITY

2.3.1.1. FISH OIL AS UNSTABLE SOURCE

As was already touched upon in Chapter 1, the appearance of lipid oxidation is a major point of concern regarding the quality of fish and fish oil as traditional n-3 LC-PUFA sources. Especially for refined fish oil, a high oxidative instability is generally observed. This is not only because of the high n-3 LC-PUFA concentration, but also due to the removal of α-tocopherol, protein residues and PLs upon refining [62]. Oxidation in this (globally important) n-3 LC-PUFA source can be slightly limited by paying attention to the environmental factors (2.1.3.2.) during production and storage: minimal exposure to oxygen, low temperature and low concentrations of transition metals. However, this is not enough and antioxidants should be added as early as possible [193]. In avoiding the use of efficient synthetic antioxidants for the above-mentioned reasons (2.2.5.1.) and with the number of approved natural antioxidants still being low, tocopherols and rosemary extracts, possessing a high content of phenolic compounds, are the most frequently used antioxidants for fish oil stabilization [16,62]. Multi-component antioxidant systems, appealing to the occurrence of synergism, are however described to be more effective. They typically consist of three components: tocopherols, ascorbic acid (or ascorbyl palmitate as lipophilic analogue) and lecithin [16,62,194]. Nevertheless, even with these preventive measures, the n-3 LC-PUFA present are still susceptible towards oxidation upon storage. Ryckebosch et al. [81] observed commercial fish oil (anchovy, mackerel and sardines blend) and tuna oil, both with an added mix of tocopherols, to be very unstable.

2.3.1.2. PHOTOAUTOTROPHIC MICROALGAE AS STABLE SOURCE

Although several microalgae are a rich source of n-3 LC-PUFA as well, the oxidative instability that is generally being observed for fish (oil) is hardly the case for certain photoautotrophic microalgal species. This oxidative stability was already mentioned in Chapter 1 as an advantage for using photoautotrophic microalgae as a novel n-3 LC-PUFA source. System wise, it was observed for both aqueous suspensions and bulk oils in former research. Gheysen et al. [8,110,195] demonstrated the oxidative stability of certain photoautotrophic species (*Nannochloropsis*, *Phaeodactylum*, *Tisochrysis* and *Porphyridium*) in contrast to *Schizochytrium* as a heterotrophic one in either microalgae enriched aqueous model systems or vegetable matrices (tomato, broccoli and carrot). Ryckebosch et al. [81] reported the oxidative stability of certain photoautotrophic microalgal oils (hexane and hexane:isopropanol extracts of *Phaeodactylum* and *Tisochrysis*) as compared to commercial n-3 LC-PUFA rich oils. In both cases the microalgae's endogenous antioxidants were pointed out as causative factor. The most important microalgal antioxidants were already highlighted in the previous section (2.2.5.) and, from these, Gheysen et al. [8] identified the importance of especially the carotenoids for obtaining oxidative stability. Based on direct antioxidant analysis, Goiris et al. [196] also indicated the significant antioxidative contribution of phenolic compounds on top of carotenoids. However, Gheysen et al. [8] showed that this contribution was only minor upon storage (indirect antioxidant analysis). A similar finding could be made for a minor influence of both antioxidative vitamins C and E [8]. Also Safafar et al. [197] reported an insignificant contribution of the latter. Although the role of these 'other microalgal antioxidants' cannot be excluded, either on their own or via synergistic effects (2.2.2. and 2.2.5.3.), whether or not influenced by environmental or system properties (2.2.3.), the findings form Gheysen et al. [8] form the starting point for the further focus on carotenoids of the current PhD research.

Interesting to remark in the light of the former autoxidation research on photoautotrophic microalgae is the fact that the influence of both structural differences in cell wall composition and processing steps like high pressure homogenization that were hypothesized to alter oxidation [198], only seemed to have a minor impact. *Nannochloropsis*, *Phaeodactylum* and *Tisochrysis* possess clear differences in cell wall structure [89,90,199], yet showed similar oxidative outcomes in the research of Gheysen et al. [8]. A high pressure homogenization of 1 pass at 100 MPa did not impact oxidative stability in the research of Gheysen et al. [200] albeit in a vegetable matrix. Therefore, although minor influences can again

not be excluded, these aspects were not explicitly examined in the current PhD research.

2.3.2. FORMER RESEARCH ON PHOTO-OXIDATIVE STABILITY

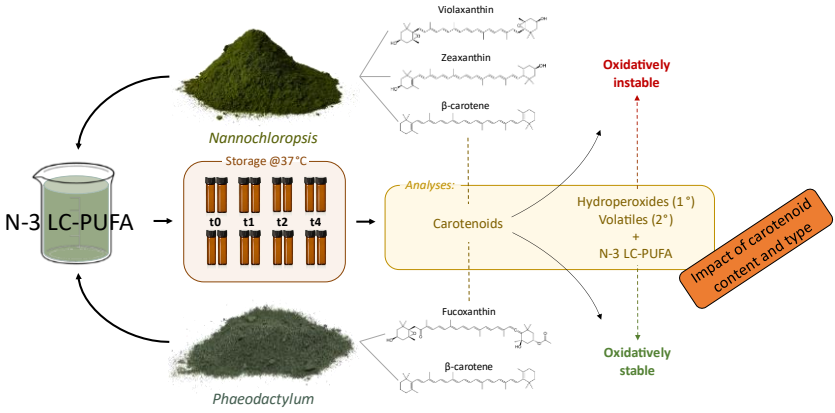
From a food research point of view, studies towards photo-oxidation are far less abundant than the ubiquitous autoxidation research. Logically, these studies cover food products that are characterized by a high transparency (e.g. nano/micro-emulsions) [201–203] and/or products in which high amounts of photosensitizers are naturally present: chlorophyll in the case of vegetable oils and seaweeds [204,205], riboflavin in the case of dairy products [206]. When specifically considering the research towards n-3 LC-PUFA rich systems, a similar diversity in photo-oxidative stability as was the case for autoxidative stability can, to the best of our knowledge, not be found. The available research is limited to O/W emulsions of fish oil ethyl esters [201] or krill oil [202], crude and refined fish liver oil [207] and dried seaweeds (*Porphyra* sp.) [205,208]. Although system diversity complicates comparison, all of these studies reported higher oxidation values upon light exposure. The photo-oxidative stability of microalgae enriched food products was not yet considered despite the presence of chlorophyll as possible photosensitizer and was thus the point of attention of Chapter 4.

PART II

EXPERIMENTAL WORK

CHAPTER 3 - OXIDATIVE STABILITY

DIFFERENCES OF AQUEOUS MODEL SYSTEMS ENRICHED WITH PHOTOAUTOTROPHIC N-3 LC-PUFA RICH MICROALGAE



3.1. INTRODUCTION

From the literature review (Part I), it could be learned that certain microalgal species have the potential of overcoming both sustainability and lipid oxidation problems when developing n-3 LC-PUFA rich food products. The first one by providing a novel source of n-3 LC-PUFA rather than overexploiting the existing ones (Chapter 1), the second by giving rise to oxidatively stable products upon food enrichment (Chapter 2). The presence of endogenous carotenoids could explain the difference between photoautotrophic microalgae giving rise to oxidatively stable products and heterotrophic microalgae leading to oxidatively unstable ones [8,110].

The current chapter aims to further investigate the importance of these endogenous carotenoids, starting with the aqueous model systems that were also considered in the research of Gheysen et al. [110]. Model systems rather than real food matrices were investigated to enable the focus on microalgal compounds. For those systems carotenoids were however not yet monitored throughout storage although this can provide information about their antioxidative role. In the absence of heat and light, carotenoid degradation can either be caused by radical scavenging or by oxidative degradation [209], preventing lipids from oxidizing respectively in a direct or indirect way. This is in contrast to the antioxidant assays giving information that is difficult to extrapolate: Müller et al. [190] observed different results depending on the method used when investigating the influence of carotenoid type on antioxidative potential using carotenoid standards. Therefore, in the current research, to obtain further insight into the antioxidative role of carotenoids upon enrichment of food products with n-3 LC-PUFA rich microalgae, a storage experiment was set up with the analysis of both lipid oxidation products (primary and secondary) and carotenoid profile throughout storage. Also the evolution of the n-3 LC-PUFA content was analyzed. With *Nannochloropsis* sp. and *Phaeodactylum* sp., two photoautotrophic n-3 LC-PUFA rich microalgae were selected which formerly showed potential for food enrichment [8,73] and which contain a clearly different carotenoid profile: *Nannochloropsis* contains various carotenoids (mostly violaxanthin, antheraxanthin, zeaxanthin and β-carotene) in varying amounts [210,211] while the profile of *Phaeodactylum* mainly consists of fucoxanthin as the major carotenoid and diadino- and/or diatoxanthin, and β-carotene as minor carotenoids [212].

3.2. MATERIALS AND METHODS

3.2.1. ENRICHED SUSPENSIONS

3.2.1.1. MICROALGAE

Freeze-dried microalgal biomass was obtained from Proviron (Hemiksem Belgium) (*Nannochloropsis* sp.) and Archimede Ricerche (Camporosso, Italy) (*Phaeodactylum* sp.). They respectively contained 42 (±2) and 20.8 (±0.5) mg n-3 LC-PUFA/g dry biomass. The carotenoid profile consisted of 3.36 (±0.03) mg violaxanthin, 0.75 (±0.02) mg zeaxanthin and 1.39 (±0.01) mg β-carotene per g dry biomass for *Nannochloropsis* and of 5.2 (±0.3) mg fucoxanthin, 0.6 (±0.1) mg diatoxanthin and 0.47 (±0.02) mg β-carotene per g dry biomass for *Phaeodactylum*. N-3 LC-PUFA and pigment content were determined upon receipt using the analyses as described below. The biomasses were stored at -80 °C until preparation of the suspensions.

3.2.1.2. PREPARATION

For each microalga the preparation of an enriched aqueous model system was performed in twofold. This preparation consisted of mixing a predetermined amount of microalgal biomass with demineralized water (1.91 g/100 g suspension for *Nannochloropsis* and 3.84 g/100 g suspension for *Phaeodactylum*) to obtain an n-3 LC-PUFA content of 80 mg n-3 LC-PUFA/100 g suspension from which a product can be claimed as being 'rich in omega-3 fatty acids' according to the European Commission [213]. In order to represent acidic food systems, the obtained suspensions were acidified to a pH of 4 using hydrochloric acid. Mixing and homogenization was performed with a lab mixer (Silverson L5M-A, East Longmeadow, U.S.A.) (2 times 5 min at 7,500 rpm) and a high-pressure homogenizer (Panda 2 K Homogenizer, GEA Niro Soavi, Parma, Italy) (1 pass at 100 MPa) for the *Phaeodactylum* suspensions. During storage, microbial growth was avoided by adding 0.25% potassium sorbate as a preservative.

3.2.2. STORAGE EXPERIMENT

3.2.2.1. SET-UP

The obtained suspensions were stored for 4 weeks at 37 °C in amber screw-capped 20 mL vials (7.5 mL suspension/vial). At this temperature the oxidation process is assumed to accelerate without changing the oxidation mechanism [148]. At four different time points (0, 1, 2 and 4 weeks) samples were taken for analysis

of primary (3.2.2.4.) and secondary (3.2.2.5.) oxidation products, fatty acid profile (3.2.2.6.) and carotenoids (3.2.2.7.). For each analysis, at each time point, 2 vials were foreseen allowing duplo analysis for each of the (in duplo) prepared suspensions. Samples were stored at -80 °C until analysis (after the end of the full storage experiment). With the exception of the secondary oxidation products, a freeze-drying step (Alpha 1-4 LDplus, Christ, Osterode am Harz, Germany) was required before analysis. Furthermore, prior to analysis of both primary oxidation products (3.2.2.4.) and fatty acid profile (3.2.2.6.), a subsequent lipid extraction (3.2.2.3.) was also necessary.

3.2.2.2. CHEMICALS

All solvents used for analyzing the samples were GC- or HPLC-grade. These included ethyl acetate, toluene (VWR, Fontenay-sous-Bois, France), acetonitrile, chloroform, hexane and methanol (Carl Roth, Karlsruhe, Germany). Used reagents were: ammonium acetate (Merck KGaA, Darmstadt, Germany), barium chloride dihydrate p.a. (Chem-Lab NV, Zedelgem, Belgium), hydrochloric acid 37% (VWR, Fontenay-sous-Bois, France), iron(II) sulfate heptahydrate ≥99% (Sigma-Aldrich, Steinheim, Germany), iron(II)chloride (Merck Schuchardt OHG, Hohenbrunn, Germany), sulfuric acid ≥95% (Acros Organics, Geel, Belgium), triphenylphosphine 99% (Sigma-Aldrich, Buchs, Switzerland) and xylenol orange disodium salt (Sigma-Aldrich, Steinheim, Germany). 2,4-Heptadienal, 2-pentenal and 2,6-nonadienal standards for the analysis of secondary oxidation products (2.2.4.) were acquired from Roth (Karlsruhe, Germany). Standards for identification and quantification of carotenoids (antheraxanthin, β-carotene, canthaxanthin, diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin) were acquired from DHI (Horsholm, Denmark).

3.2.2.3. LIPID EXTRACTION

For the analyses of both primary oxidation products and fatty acid profile, a preceding total lipid extraction was required. This was performed with chloroform:methanol (1:1) according to the method of Ryckebosch et al. [108]. In this, 100 mg freeze-dried suspension was subjected to two serial chloroform:methanol (1:1) extractions, each one covering the following steps:

- Addition of 4 mL methanol, 2 mL chloroform, 0.4 mL water and an additional 2 mL chloroform and 2 mL water after a 30 s vortex step
- Phase separation after vortexing (30 s) and centrifugation (10 min, 750 g) (SL 16 Centrifuge, Thermo Scientific) in which the upper aqueous phase was disposed and the lower organic phase was kept aside

- Re-extraction of the remaining pellet with 4 mL chloroform:methanol (1:1), followed by vortexing, centrifugation and organic phase collection

All extracts were pooled and subsequently dried over sodium sulfate using a Whatman 1 filter and washing with chloroform:methanol (1:1). The solvent was removed by rotary evaporation at 40 °C.

3.2.2.4. PRIMARY OXIDATION PRODUCTS

The PV as a measure for primary oxidation products (hydroperoxides) was determined on the total lipid extract of the samples according to the adapted spectrophotometric ferrous oxidation xylenol orange (FOX) method of Gheysen et al. [156]. Also the described triphenylphosphine (TPP) correction to account for possible interferences was implemented. Briefly, a PV value 'without TPP' and a PV value 'with TPP' were determined as follows: 10 mg of the extracted lipids were dissolved in 9.9 mL chloroform:methanol (7:3) and diluted 1/10 to the same volume, in which 1 mL of 10 mM TPP was incorporated for the PV value 'with TPP' followed by at least 15 min rest to allow the hydroperoxides to react with TPP. A sample absorbance (A_S) was determined by adding both 50µl xylenol orange solution (10 mM) and 50 µl acidic iron(II)chloride solution (18 mM, acidified with 2% HCl 10M) and measuring the absorbance after exactly 5 min at 560 nm. This value was corrected by both the blank absorbance of the sample at 560 nm ($A_{B,S}$) and the blank of the iron(II)solution determined by measuring absorbance at 5 min after adding both xylenol orange and iron(II)chloride solution to the pure 9.9 mL chloroform:methanol (7:3) solvent in the same quantities ($A_{B,Fe(II)}$). The corrected absorbance ($A_S - A_{B,S} - A_{B,Fe(II)}$) was converted to a corresponding iron(III) content (in µg) by setting up an iron(III) calibration curve with an iron(III)chloride standard solution. Sample series of which the absorbance fell out of the range of the calibration curve were diluted more than the stated 1/10. The PV value (in meq/kg lipids) could subsequently be calculated by the following equation in which W is the amount of lipids (in g), d is the dilution, 55.84 is the atomic mass of iron (in g/mol) and 2 is the amount of iron atoms that can be oxidized by a single hydroperoxide:

$$PV\ (meq/kg\ lipids) = \frac{amount\ of\ iron(III)\ (in\ \mu g)}{W \times d \times 55.84 \times 2}$$

The interference corrected PV value could then be calculated by subtracting the PV value 'with TPP' from the one 'without TPP'. To regard the oxidation in terms of suspension quantities, the value was expressed in meq/100 g suspension, and

finally, to focus on the impact of oxidation throughout storage, the PV data was expressed as delta values, indicating the relative increase or decrease compared to the start of the storage experiment (day zero) for both suspensions.

3.2.2.5. SECONDARY OXIDATION PRODUCTS

The volatile secondary oxidation products were analyzed using HS-SPME Arrow GC-MS. A GC Trace 1300 gas chromatograph coupled with an ISQ 7000 single quadrupole mass spectrometer (Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium) was used. Prior to sample analysis, method settings were optimized. The samples were incubated at 60 °C for 15 min, and the volatile compounds were extracted on a PAL DVB/CAR/PDMS SPME Arrow 1.1 mm fiber (CTC Analytics, Zwingen, Switzerland) for an additional 15 min at 60 °C. These compounds were subsequently injected on a Rxi-1ms column (50 m length, 0.20 mm ID, 0.33 µm film) (Restek, U.S.A.), with a 1/50 split ratio, helium as a carrier gas, and a desorption temperature of 250 °C for 2 min. The following time-temperature program was used: 40 °C (5 min), 40-250 °C (5 °C/min), 250 °C (5 min). Transfer lines were kept at 280 °C. For mass spectroscopy, electron ionization at 70 eV was applied and mass units in the range of 40-250 amu were scanned. Data was monitored and processed with Chromeleon 7 Chromatography Data System software (Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium). For the identification of volatile compounds, the NIST MS Search 2.3 spectral library was used. From the identified compounds, specific markers originating from n-3 LC-PUFA oxidation [7,161,163,214] for which the identity could be confirmed by the use of standards, were selected: 2,4-heptadienal, 2-pentenal and 2,6-nonadienal. Volatile compound contents (in µg/100 g suspension) were calculated using calibration plots of the standards spiked in an acidic aqueous suspension and again expressed as delta values, indicating the relative increase or decrease compared to day zero for both suspensions. This method can be considered as semi-quantitative. A more extensive discussion on the implementation of this method can be found in the appendix of this PhD.

3.2.2.6. FATTY ACID PROFILE

The fatty acid profile, among which n-3 LC-PUFA, was determined by GC after an acid-catalyzed methyl transesterification of the total lipid extract using the method as described by Gheysen et al. [110]. In short, 5 mg of the extracted lipids was dissolved in 1 mL toluene, and 2 mL 1% sulfuric acid in methanol was added. After airtight overnight storage at 50 °C and addition of 5 mL aqueous sodium chloride solution (5%), the formed fatty acid methyl esters (FAMEs) were

extracted with 3 mL hexane and diluted to a concentration of ca. 500 ng/µL. GC analysis was performed with a Trace GC Ultra (Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium) equipped with cold-on-column injection, an EC Wax column (30 m length, 0.32 mm ID, 0.25 µm film) (Grace, Lokeren, Belgium) and flame ionization detection (FID). The following time-temperature program was used: 70–180 °C (10 °C/min), 180–235 °C (4 °C/min), 235 °C (4.75 min). Peak identification was possible using an external standard (Nu-Check Prep, Elysian, U.S.A.). Chromecard 2.5 for Windows software (Interscience, Louvain-la-Neuve, Belgium) was used for peak area determination. As a measure for n-3 LC-PUFA instability, the ratio of the n-3 LC-PUFA content over the content of palmitic acid (C16:0), a thermally and oxidatively stable fatty acid abundantly present in both *Nannochloropsis* and *Phaeodactylum*, was calculated and further reported. It was normalized to a starting value of 1 to improve comparison throughout storage across different suspensions.

3.2.2.7. CAROTENOIDS

Identification and quantification of carotenoids was performed by high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection after extraction with acetone:methanol (7:3). The method as described by Gheysen et al. was used [8]. In short, 25 mg freeze-dried suspension was subjected to four sequential extraction steps, each consisting of: addition of 5 mL acetone:methanol (7:3), vortexing (30s), centrifugation (10 min, 750 g) and extract collection. Glass beads were used to improve extraction and the extracts were pooled. After filtering with PVDF syringe filters (4 mm, 0.2 µm), the samples were placed on a HPLC (Alliance e2695, Waters, Zellik, Belgium) equipped with PDA (Waters 996, Zellik, Belgium) and a Nova Pak C18 column 60A (4 µm; 3.9 mm x 150 mm) at 30 °C, in which they were kept at 10 °C before injection. Gradient elution was applied with methanol:ammonium acetate buffer (0.5 M, pH 7.2) (8:2) [A], acetonitrile:milliQ water (9:1) [B] and ethyl acetate [C] as mobile phases, and this with the following program (all linear gradients):

- **0-3 min:** 100% A to 100% B
- **3-14 min:** 100% B to 50% B and 50% C
- **14-16 min:** 50% B and 50%C to 50% A and 50% C
- **16-22.5 min:** 50% A and 50% C to 20% A and 80% C
- **22.5-26 min:** isocratic
- **26-30 min:** 20% A and 80% C to 100% C
- **30-32 min:** 100% C to 80% A and 20% C
- **32-33 min:** 80% A and 20% C to 100% A

Peak identification was based on retention time and UV spectrum of the following standards: antheraxanthin, β-carotene, canthaxanthin, diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin. The peak area was quantified at 436 nm with Empower 2 for Windows software, and calculated to an absolute content of the identified pigments by setting up calibration curves with the standards. For the *Phaeodactylum* systems, diatoxanthin could not be quantified throughout storage because of an overlapping peak of a chlorophyll breakdown product after processing. Therefore, fucoxanthin (as major carotenoid) and β-carotene (as minor carotenoid) were further focused upon for those systems.

3.2.3. STATISTICAL ANALYSIS

Statistical analysis was performed with the use of JMP Pro 16.0.0 software (SAS Institute Inc., Cary, USA). A significance value of 0.05 was used. For all analyzed compounds the impact of storage time was statistically evaluated with one-way analysis of variance (ANOVA) and a post hoc Tukey test. In this way the time point at which a relevant increase or decrease occurred could be determined. Furthermore, for the n-3 LC-PUFA/C16:0 ratio and the β-carotene content, significant differences between the *Nannochloropsis* and the *Phaeodactylum* suspensions were investigated at each time point, also with one-way ANOVA. This additional statistical evaluation was not performed for the primary and secondary oxidation compounds, because these compounds can both increase and decrease throughout storage making a single timepoint comparison irrelevant.

3.3. RESULTS AND DISCUSSION

3.3.1. PRIMARY OXIDATION

Figure 3.1 shows the results of the primary lipid oxidation. For both *Nannochloropsis* and *Phaeodactylum* suspensions a significant increase in hydroperoxides throughout storage could be observed. However, with a ΔPV value of 0.07 (±0.03) meq hydroperoxides/100 g suspension after 4 weeks of storage, this increase was clearly higher for the *Nannochloropsis* enriched suspension than for the *Phaeodactylum* enriched one, which only increased towards 0.023 (±0.004) meq hydroperoxides/100 g suspension. Considering the magnitude of these increases, primary oxidation in the *Phaeodactylum* system could be regarded as low since it was comparable with the increase of oxidatively stable systems in former research [110], whereas the *Nannochloropsis* system indicated primary lipid oxidation to a certain degree.

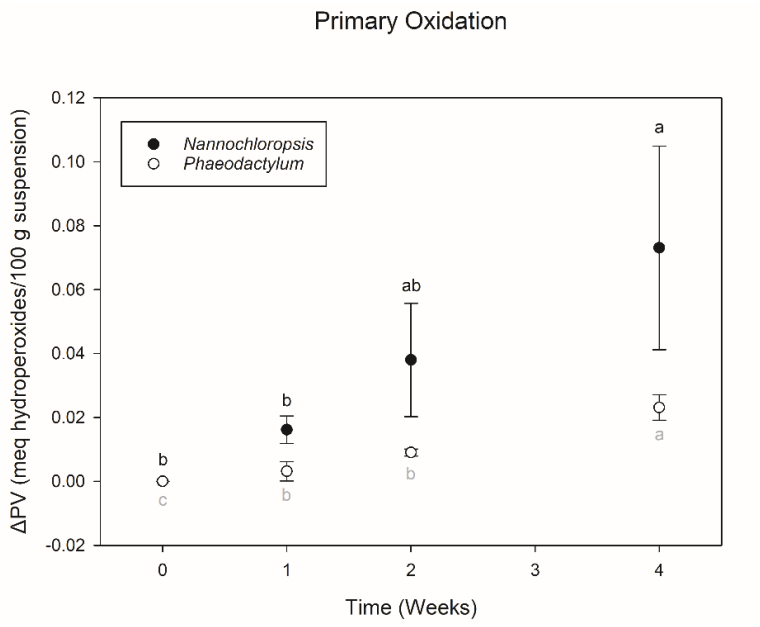


Figure 3.1: Primary oxidation of the *Nannochloropsis* (black) and *Phaeodactylum* (white) enriched suspensions during 4 weeks of storage at 37 °C. The PV (mean ± SD, n = 2 x 2), expressed as delta values in meq hydroperoxides/100 g suspension, representing the relative increase or decrease compared to day zero. Different letters indicate statistical differences (α=0.05) within each system (*Nannochloropsis* in black or *Phaeodactylum* in grey).

3.3.2. SECONDARY OXIDATION

Figure 3.2 shows the results of the secondary lipid oxidation products. While the primary oxidation products showed a clear difference between the *Nannochloropsis* and *Phaeodactylum* suspensions, the secondary oxidation products, which are formed out of the primary ones as a further step in the oxidation process [7], did not. For both microalgae all three compounds (that were selected as specific markers for the secondary oxidation of n-3 LC-PUFA) did not show remarkable in- or decreasing trends throughout the storage experiment. For the *Nannochloropsis* suspensions, 2,4-heptadienal could even not be detected on the further time points. Some significant differences between different time points were determined, but these seemed to be of minor relevance given the absence of clear oxidation tendencies.

3.3.3. N-3 LC-PUFA EVOLUTION

Figure 3.3 shows the evolution of n-3 LC-PUFA expressed as the ratio of n-3 LC-PUFA over C16:0. Since this ratio was different for the *Nannochloropsis* and the *Phaeodactylum* suspension at the start of the storage experiment (1.23 ±0.06 and 1.6 ±0.1 respectively), the data were normalized to a starting value of 1 to be able to better compare the evolution throughout storage in both suspensions. Both suspensions showed a decrease, but while the ratio remained fairly stable for the *Phaeodactylum* suspension (apart from a significant decrease at week 1), it clearly decreased throughout storage for the *Nannochloropsis* suspension. After 4 weeks, the (normalized) ratio was significantly lower for the *Nannochloropsis* suspension (0.5 ±0.2) than for the *Phaeodactylum* suspension (0.86 ±0.03). Based on this ratio, it could be stated that for *Nannochloropsis* approximately half of the n-3 LC-PUFA (intended for product enrichment) degraded, which is of course undesirable.

A decrease in n-3 LC-PUFA content can be caused by thermal degradation, physical losses or lipid oxidation [215]. With thermal degradation occurring at significantly higher temperatures than the storage temperature of 37 °C [216,217] and physical losses being out of question since the ratio of n-3 LC-PUFA over C16:0 was considered, lipid oxidation must be the cause for the observed n-3 LC-PUFA degradation.

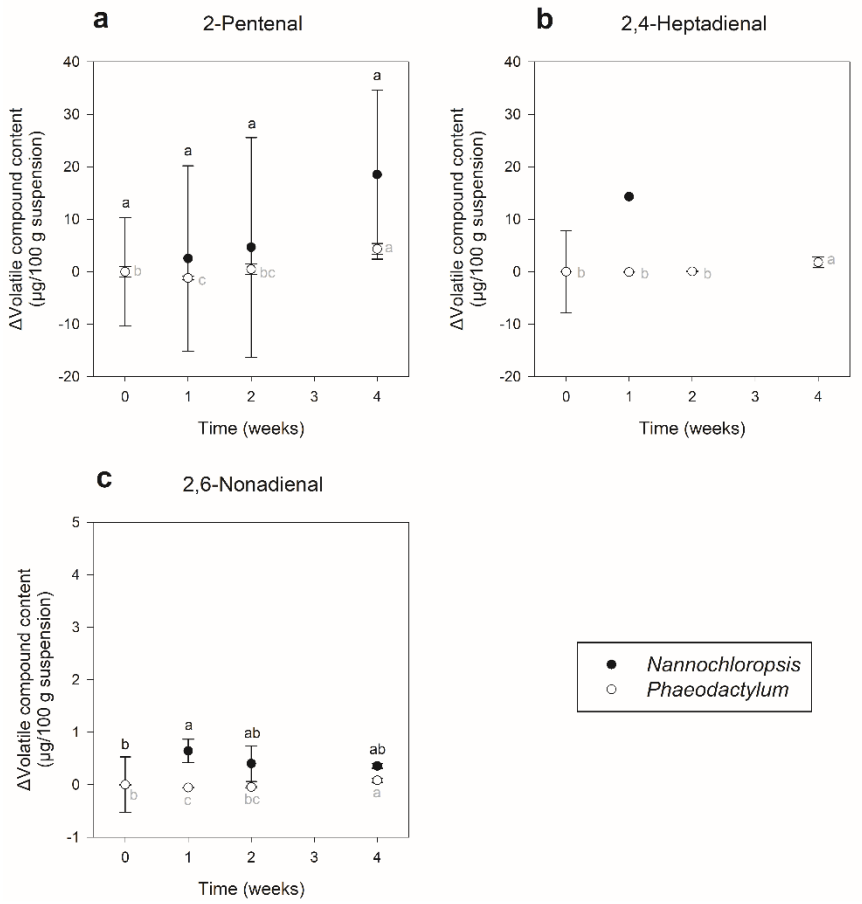


Figure 3.2: Secondary oxidation of the *Nannochloropsis* (black) and *Phaeodactylum* (white) enriched suspensions during 4 weeks of storage at 37 °C. The content of specific volatile compounds originating from n-3 LC-PUFA oxidation [2-pentenal (a), 2,4-heptadienal (b) and 2,6-nonadienal (c)] (mean ± SD, n = 2 x 2), expressed as delta values in µg/100 g suspension, representing the relative increase or decrease compared to day zero. Different letters indicate statistical differences (α=0.05) within each system (*Nannochloropsis* in black or *Phaeodactylum* in grey).

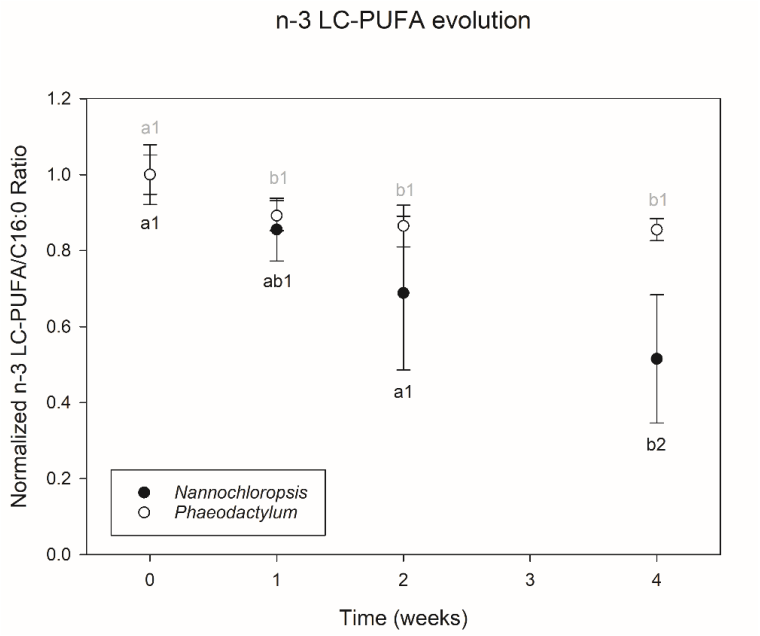


Figure 3.3: n-3 LC-PUFA evolution in terms of the n-3 LC-PUFA/C16:0 ratio of the *Nannochloropsis* (black) and *Phaeodactylum* (white) enriched suspensions during 4 weeks of storage at 37 °C. For both suspensions, the ratio (mean ± SD, n = 2 x 2) was normalized to a value of 1 at the start of the storage experiment. Different letters indicate statistical differences (α=0.05) within each system (*Nannochloropsis* in black or *Phaeodactylum* in grey) and statistical differences (α=0.05) at a specific time point between the two microalgae are indicated by a different number.

3.3.4. OXIDATIVE STABILITY

By combining the observations of the above lipid oxidation related analyses, either measuring lipid oxidation products (primary and secondary) or loss of lipid oxidation substrate (n-3 LC-PUFA), conclusions can be drawn on oxidative stability. The analyses of primary oxidation products (3.3.1) and n-3 LC-PUFA (3.3.3) point in the same direction: the *Nannochloropsis* enriched suspension was oxidatively unstable (given its relatively higher increase in primary oxidation products and its clear degradation of n-3 LC-PUFA), whereas the *Phaeodactylum* enriched suspension was (quite) oxidatively stable (considering its limited increase in primary oxidation products and its relatively stable course of the n-3 LC-PUFA/C16:0 ratio throughout storage). Also the fact that both analyses showed high standard deviations for the further time points of the *Nannochloropsis* suspension indicated its oxidative instability: due to the independent storage in separate vials, one sample can be located somewhat

further in the autocatalytically propagated process of lipid oxidation than the other one, which is a variation that would not be observed if lipid oxidation was not occurring.

Secondary oxidation products (3.3.2) on the other hand were not indicative of the observed stability differences. Although the absence of a clear increase in secondary oxidation products was consistent with the oxidative stability of the *Phaeodactylum* system, this was not the case for the *Nannochloropsis* system in which an increase would be more accordant to the oxidatively unstable outcome. One explanation could be that the majority of secondary oxidation compounds still needs to be formed out of the primary oxidation products. However, the fact that 2,4-heptadienal increased after one week of storage but was absent thereafter is in that case still remarkable. Another explanation could be a further reaction of secondary lipid oxidation products, leaving their formation unnoticed. Although further oxidation pathways are diverse [7,137], one reaction pathway that can be thought of since it was previously described for n-3 LC-PUFA rich sources [83,218] is the formation of so-called 'Strecker aldehydes'. These are formed throughout a reaction between lipid oxidation products (more specifically hydroxyalkenals, epoxyalkenals and unsaturated epoxy keto fatty acids originating from secondary lipid oxidation products) and primary amine groups of amino acids or phosphatidylethanolamine (PE) [219]. Their appearance was shown in former research studying the oxidation of krill oil which is known to contain both PE and amino acid residues [220]. Since amino acids and PE are present in *Nannochloropsis* as well [221], Strecker degradation reactions may also occur in the current study. Therefore the presence of Strecker degradation products was investigated by means of GC-MS (volatiles). 2-Methylbutanal, 3-methylbutanal, 2-methyl-2-pentenal and 2-methyl-2-butenal, four volatiles which were reported as Strecker degradation products in marine PL systems by Lu et al. [219], were all found to be present in increasing amounts throughout storage (data not shown). This did coincide with the occurrence of lipid oxidation. However, it should be noted that Strecker aldehydes can also be formed by enzymatic pathways [222] so their presence should not be utilized as a measure for lipid oxidation without assessing other oxidation parameters.

The evaluation of n-3 LC-PUFA on the other hand has, despite its claimed insensitivity [223], proven to be a good indicator for lipid oxidation since their degradation could only be explained by lipid oxidation in the current research. When assessing lipid oxidation in microalgae enriched products, analyzing

n-3 LC-PUFA on top of (i.e. not instead of) the lipid oxidation products would therefore be a good practice to evaluate oxidative stability.

3.3.5. CAROTENOIDS

Given the earlier discussed importance of endogenous carotenoids for oxidative stability in n-3 LC-PUFA rich microalgae [8,110,195] and the known difference in the carotenoid profile of *Nannochloropsis* and *Phaeodactylum*, a difference in oxidative stability of their suspensions (as was discussed in 3.3.4.) is an interesting starting point for getting further insight into the antioxidative role of carotenoids. First of all their presence and evolution throughout storage is being discussed (3.3.5.1.) after which the association with lipid oxidation is being investigated (3.3.5.2).

3.3.5.1. CAROTENOID CONTENT THROUGHOUT STORAGE

Figure 3.4 shows the content of the different carotenoids throughout storage in both of the enriched suspensions. At the start of the storage experiment, the *Nannochloropsis* enriched suspension consisted of 1.53 (±0.03) mg zeaxanthin, 6.1 (±0.1) mg violaxanthin and 2.32 (±0.03) mg β-carotene per 100 g suspension, making up a total of 10.0 (±0.1) mg carotenoids/100 g suspension. For the *Phaeodactylum* enriched suspension, the initial β-carotene content was quite similar (2.19 (±0.02) mg/100 g suspension), but with 28 (±2) mg fucoxanthin/100 g suspension the carotenoid profile showed a clear difference in composition as well as in quantity (30 (±2) mg carotenoids/100 g suspension). As was mentioned in the 'Materials and Methods' section (3.2.2.7.), diatoxanthin was, although being slightly present in the *Phaeodactylum* biomass (see 3.2.1.1.), not considered in this because of quantification no longer being possible after processing due to an overlapping chlorophyll breakdown product peak. While for the *Nannochloropsis* enriched suspension the time zero content of the different carotenoids was in line with the ones that were expected based on the predetermined biomass composition and the biomass addition quantities (see 3.2.1.), this was not the case for the *Phaeodactylum* enriched suspension. Especially for fucoxanthin, it showed higher values than expected: 28 (±2) mg/100 g suspension instead of 20 (±1) mg/100 g suspension. The reason for this deviation could be that the extraction solvent was not capable of extracting all carotenoids when analyzing the pure biomass, while it was for the suspensions that went through processing (suspension and high pressure homogenization) and freeze-drying. Investigating the *Phaeodactylum* biomass, the degree of cell (wall) disruption, facilitating extraction upon higher values, was indeed extremely

low as compared to former research (hexane:isopropanol extraction efficiency of 19%) [81,198].

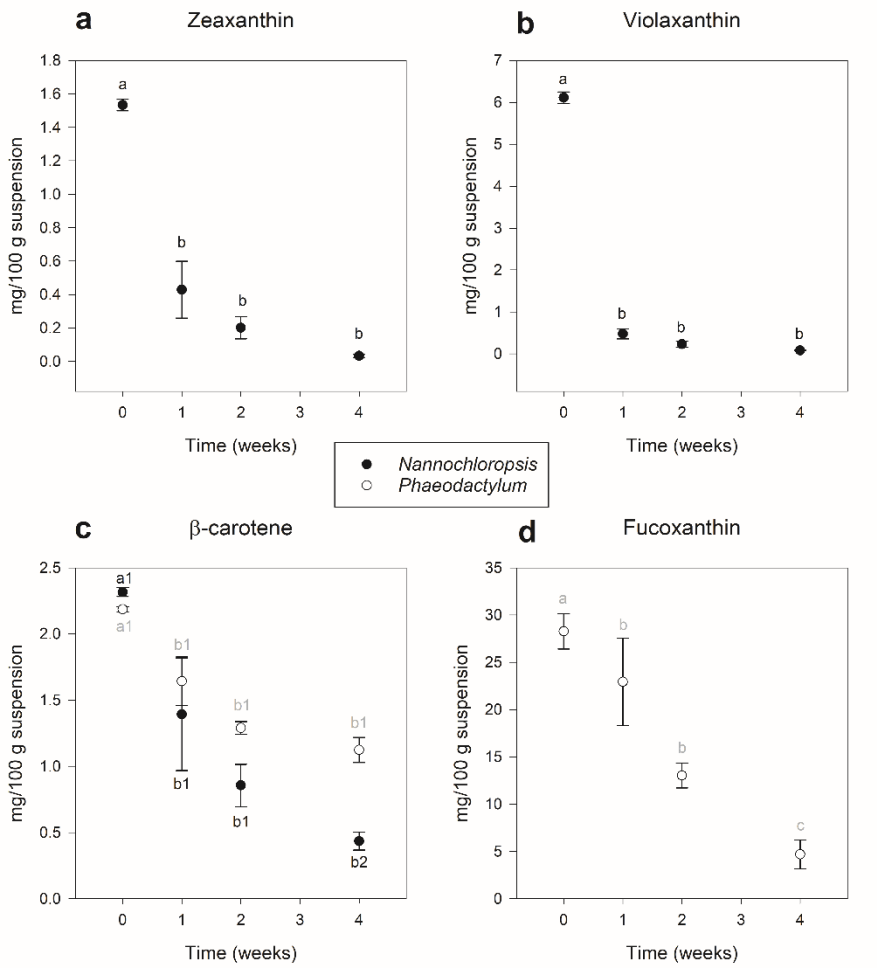


Figure 3.4: Evolution of zeaxanthin (a), violaxanthin (b), β-carotene (c) and fucoxanthin (d) content in mg/100 g suspension (mean ±SD, n = 2 x 2) of the *Nannochloropsis* (black) and *Phaeodactylum* (white) enriched suspensions during 4 weeks of storage at 37 °C. For each carotenoid, different letters indicate statistical differences (α=0.05) within each system (*Nannochloropsis* in black or *Phaeodactylum* in grey) and statistical differences (α=0.05) at a specific time point between the two microalgae are indicated by a different number.

For all different carotenoids a significant degradation throughout storage could be observed. However, the degree to which this occurred seemed to differ between different carotenoids and algae. Both zeaxanthin and violaxanthin (only

present in the *Nannochloropsis* suspensions) showed the most extensive degradation: their content was already drastically lower after 1 week of storage (with a degradation of respectively 72 (±11)% and 92 (±2)%), and was almost zero at the end of the storage experiment (with respectively only 2 (±1)% and 1.3 (±0.2)% of the initial content remaining). Fucoxanthin and β-carotene on the other hand showed a more gradual and incomplete degradation. Fucoxanthin (only present in the *Phaeodactylum* suspension) degraded for 19 (±17)% after 1 week of storage and for 83 (±5)% at the end of the storage experiment. For β-carotene, which was the only carotenoid being present in both *Nannochloropsis* and *Phaeodactylum* suspensions, the degradation after 1 week of storage was also more moderate than for zea- and violaxanthin (for *Nannochloropsis* as well as *Phaeodactylum*), but a significant difference after the full storage experiment could be observed between both microalgae. While for *Nannochloropsis* 81 (±3)% of the initial β-carotene degraded, only 49 (±4)% did for the *Phaeodactylum* system. Regarding the total amount of carotenoids at the end of the storage experiment, the *Nannochloropsis* suspension contained 0.55 (±0.07) mg carotenoids/100 g suspension, which was approximately tenfold lower than for the *Phaeodactylum* suspension containing 6 (±2) mg carotenoids/100 g suspension.

3.3.5.2. ANTIOXIDATIVE ROLE

In the current research, the degradation of carotenoids illustrates their antioxidative role. Since storage was performed in dark conditions and at a moderate temperature of 37 °C, mechanisms for carotenoid breakdown are either radical scavenging or oxygen degradation [209]. As radical scavenging prevents lipid radicals from propagating the lipid oxidation process and oxygen degradation depletes oxygen in the near environment of lipids, both can be perceived as being antioxidative. Therefore, given that all carotenoids in the current research showed a significant degradation (3.3.5.1.), it could be stated that all carotenoids acted as an antioxidant. However, looking at the earlier described differences in oxidative stability (3.3.4), this antioxidative activity did not suffice for keeping the *Nannochloropsis* enriched suspension oxidatively stable. Therefore, although the research of Gheysen et al. [110] suggested that the presence of carotenoids in microalgal suspensions is more important for oxidative stability than the specific type and amount (considering the thorough lipid oxidation in *Schizochytrium* not containing any carotenoids), the results of the current research nuanced this statement. Carotenoid content and type do seem to play their role as illustrated by the differences between *Nannochloropsis* and *Phaeodactylum*.

The impact of carotenoid content seemed to be the most decisive one. The *Phaeodactylum* suspension, showing oxidative stability, had an initial carotenoid content of 30 (±2) mg carotenoids/100 g suspension, which was three times higher than the one of the oxidatively unstable *Nannochloropsis* suspension. Also, at the end of the storage experiment, the *Phaeodactylum* suspension contained 6 (±2) mg carotenoids/100 g suspension, which was still above twice the content of the *Nannochloropsis* suspensions after only one week of storage (2.3 (±0.5) mg carotenoids/100 g suspension).

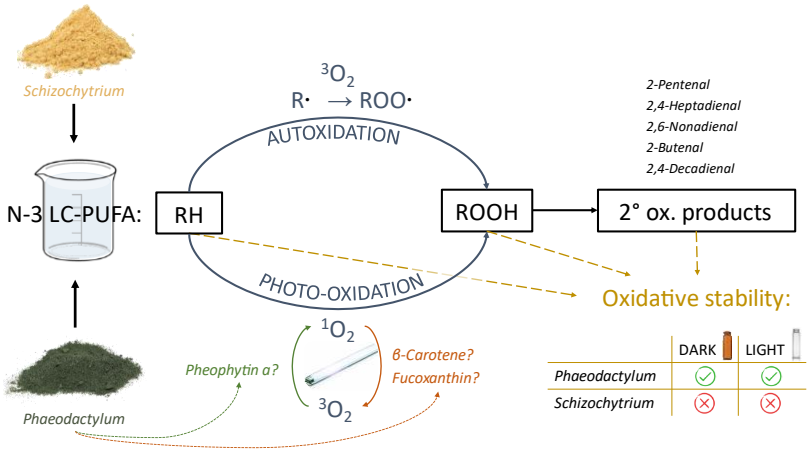
Regarding the impact of carotenoid type, it might be that a different carotenoid composition (zeaxanthin, violaxanthin and β-carotene for *Nannochloropsis*, and fucoxanthin and β-carotene for *Phaeodactylum*) led to a difference in oxidative stability. Apart from that, the difference in degradation degree could be mentioned. The fact that zea- and violaxanthin showed a faster and more extensive degradation than β-carotene and fucoxanthin indicates a different interaction with the oxidation process. It seemed that for the *Nannochloropsis* suspension, although insufficient for keeping it stable, violaxanthin and zeaxanthin acted as early antioxidants, while β-carotene was lagging behind. Given the higher apolarity of β-carotene, this might be a localization related effect. After all localization, which is partly associated with polarity, is known to be an important factor in determining antioxidative activity in heterogeneous systems [224].

It is however unclear to what extent quantity plays a role in explaining the carotenoid type effect. It is for example hard to state whether fucoxanthin is a more potent antioxidant than viola- or zeaxanthin. The question could be raised whether violaxanthin and zeaxanthin showed a fast degradation because of the initial carotenoid content already being relatively low, or (in other words) whether they would show a comparable more gradual degradation and antioxidative activity if they would start at the higher content like fucoxanthin did in the *Phaeodactylum* suspension.

3.4. CONCLUSIONS

In conclusion it can be stated that despite the presence of endogenous carotenoids (formerly shown to be of importance for oxidative stability), aqueous systems enriched with photoautotrophic n-3 LC-PUFA rich microalgae can show clear differences in oxidative stability. Enrichment with *Phaeodactylum* led to oxidatively stable products, confirming its enrichment potential, whereas using *Nannochloropsis* showed clear lipid oxidation. This was indicated by the results of both primary oxidation and n-3 LC-PUFA evolution. Regarding the antioxidative role of the endogenous carotenoids, all carotenoids exerted an antioxidative effect as shown by their degradation, but for the *Nannochloropsis* suspension this did not suffice for preventing lipid oxidation. Rather than the presence of carotenoids on its own, also their content (initially being threefold higher in the oxidatively stable *Phaeodactylum* enriched suspensions) has an impact. Furthermore the carotenoid type might be of importance, as indicated by the difference in profile and degradation trend, although the contribution of quantity to these differences remains unclear.

CHAPTER 4 - PHOTO- VS. AUTOXIDATIVE STABILITY OF AQUEOUS MODEL SYSTEMS ENRICHED WITH N-3 LC-PUFA RICH MICROALGAE



Adapted from: Demets, R., Bonneux, L., Dejonghe, C., Gheysen, L., Van Loey, A., & Foubert, I. (2022). Photo-Oxidative Stability of Aqueous Model Systems Enriched with Omega-3 Long-Chain Polyunsaturated Fatty Acid-Rich Microalgae as Compared to Autoxidative Stability. *Journal of Agricultural and Food Chemistry*, *70* (18), 5691–5700.

4.1. INTRODUCTION

The literature review (Part I) showed that photoautotrophic n-3 LC-PUFA rich microalgae have the potential for developing oxidatively stable n-3 LC-PUFA rich food products as a sustainable alternative for fish (oil). This with the oxidative stability being due to the presence of endogenous carotenoids. The previous chapter (Chapter 3) brought some nuance by showing that, on top of this presence, carotenoid content and (possibly) type also matter.

All of the oxidation research on food enrichment with n-3 LC-PUFA rich microalgae so far has, to the best of our knowledge, focused on autoxidation. Since more consumers are demanding transparent packages giving them the chance to visually evaluate the product quality [225], the possibility of photo-oxidation in food products stored in transparent containers under illuminated conditions cannot be ignored. Whereas the transmission of UV light can be limited depending on the transparent packaging material used [7], the transmission of visible light cannot. However, visible light requires the presence of photosensitizers to induce lipid oxidation [140]. Like was touched upon in Chapter 2, chlorophyll is such a (type II) photosensitizer that is, in addition, abundantly present in photoautotrophic microalgae. Therefore, the potential of using (autoxidatively stable) photoautotrophic microalgae for developing stable n-3 LC-PUFA rich food products that can be stored in transparent containers is unsure. The current chapter aims to bridge this uncertainty.

Moreover, carotenoids can again be of importance regarding photo-oxidative stability, which also makes it interesting to consider photo-oxidation from the larger focus on carotenoids in the current PhD research. As was mentioned in Chapter 2 as well, carotenoids have the ability to inhibit photo-oxidation by either bringing the excited photosensitizer or singlet oxygen back to their ground state (physical quenching) or by forming stable products in reaction with singlet oxygen (chemical quenching) [7]. It is however unclear whether the endogenous carotenoids suffice for obtaining oxidative stability as it is reported that their quenching capacity can be exceeded if the microalgae are photosynthetically inactive [226], as they would be when enriching food products.

To examine if autoxidatively stable photoautotrophic microalgae can retain their enrichment potential under illuminated conditions, *Phaeodactylum* was selected. It contains both chlorophylls as possible photosensitizers and carotenoids as possible quenchers, and was shown to be autoxidatively stable in the previous chapter. To evaluate the extent to which illumination promotes lipid oxidation,

also *Schizochytrium*, a heterotrophic microalga showing strong autoxidation upon enrichment [8,110] and lacking both chlorophylls and carotenoids, was considered as a positive control for strong lipid autoxidation. To avoid interaction with other food compounds, acidic aqueous model systems (representing acidic food matrices) were still studied as a first step in estimating the possible effect of photo-oxidation. To investigate the oxidative stability, the enriched systems were exposed to an accelerated storage experiment of 28 days at 37 °C in both dark and illuminated conditions, throughout which the n-3 LC-PUFA content, PV (primary oxidation products), volatile compounds (secondary oxidation products) and pigments were analyzed.

4.2. MATERIALS AND METHODS

4.2.1. MATERIALS

4.2.1.1. SOLVENTS AND REAGENTS

The same solvents and reagents were used as those described in section 3.2.2.2.

4.2.1.2. MICROALGAL BIOMASS

Freeze-dried *Phaeodactylum* sp. biomass was obtained from Archimede Ricerche (Camporosso, Italy). It had an n-3 LC-PUFA content of 20.8 (±0.5) mg/g dry biomass and the pigment profile per g dry biomass consisted of 5.2 (±0.3) mg fucoxanthin, 0.6 (±0.1) mg diatoxanthin, 0.47 (±0.02) mg β-carotene, 3.0 (±0.3) mg chlorophyll a, 2.2 (±0.1) mg chlorophyll c2 and 0.67 (±0.06) mg pheophytin a. Spray-dried *Schizochytrium* sp. biomass was obtained from Bunge (Sant Just Desvern, Spain) and had an n-3 LC-PUFA content of 256 (±25) mg/g dry biomass. N-3 LC-PUFA and pigment content were determined using the analyses as described below. Both microalgal biomasses were stored at -80 °C until further use.

4.2.2. EXPERIMENTAL SET-UP

Figure 4.1 shows a schematic overview of the experimental set-up. It consists of the preparation of aqueous model suspensions enriched with either *Phaeodactylum* or *Schizochytrium* biomass, followed by the storage experiment in dark or illuminated conditions.

4.2.2.1. SUSPENSION PREPARATION

For the preparation of the suspensions, microalgal biomass was added to demineralized water in such an amount that an n-3 LC-PUFA content of 80 mg/100 g suspension was reached (3.84 g/100 g suspension for *Phaeodactylum* and 0.31 g/100 g suspension for *Schizochytrium*). This is the amount from which a product can be claimed as 'rich in omega-3 fatty acids' in the European Union [213]. Subsequentially, a mixing step of two times 5 min at 7,500 rpm was applied with a lab mixer (Silverson L5M-A, East Longmeadow, U.S.A.). The suspensions were acidified with hydrochloric acid to a pH of 4, representing acidic food products like fruit based systems. To obtain physically stable suspensions, a high pressure homogenization step of 1 pass at 100 MPa was implemented with a Panda 2K Homogenizer (GEA Niro Soavi, Parma, Italy), in which an excessive temperature

increase was avoided by using a cryostat (4 °C) (Haake, Karlsruhe, Germany) at the inlet and by collecting the homogenized suspensions in an ice bath cooled recipient. Microbiological stability was assured by adding 0.25% potassium sorbate before storage. Throughout the preparation of the suspensions, light incidence was avoided. For both *Phaeodactylum* and *Schizochytrium*, the suspensions were prepared in twofold.

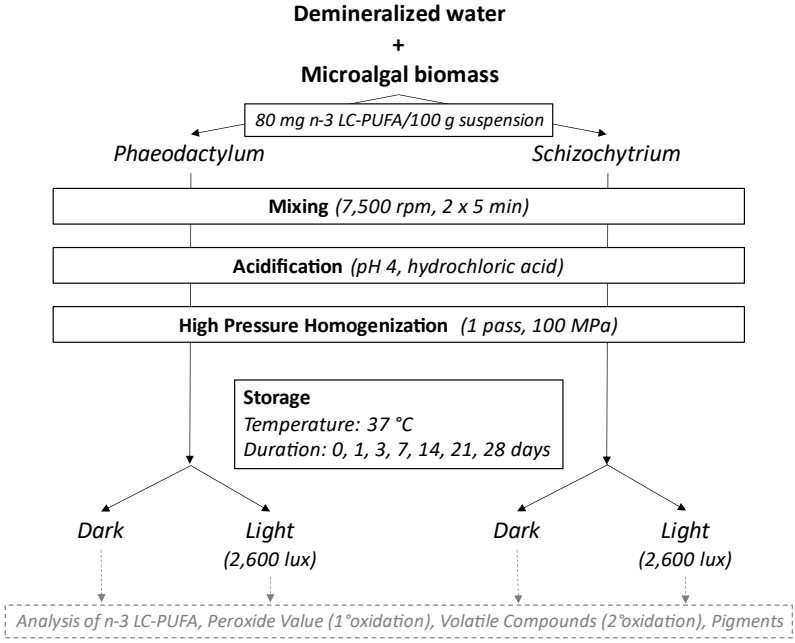


Figure 4.1: Experimental set-up

4.2.2.2. STORAGE EXPERIMENT

For the storage experiment, 7.5 mL of the suspensions was transferred to amber (dark set-up) or clear (light set-up) screw-capped 20 mL vials. Both were stored in a temperature controlled room for 28 days at 37 (±1) °C. This temperature is assumed to accelerate the process of lipid oxidation without changing the reaction mechanisms [148], allowing to determine the evolution of lipid oxidation within a reasonable time frame. Analysis of n-3 LC-PUFA content (by measuring the fatty acid profile), primary oxidation products (by measuring PV), secondary oxidation products (by measuring volatile compounds) and pigment content was performed for seven different time points (day 0, 1, 3, 7, 14, 21, 28). The methods

used for the different analyses are described in the section below. During storage, the clear vials were exposed to fluorescent light (Philips Master TL5 HE 35W/840, Holland) (light set-up). An average light intensity of ca. 2,600 lux at the surface was measured using an STS-VIS Miniature Spectrometer (Odean Optics, Dunedin, U.S.A.), which is an intensity comparable to the highest food store light intensities. To cancel light intensity variations across the lamp, the samples were randomly distributed and rearranged two times in between each of the different time points. The amber vials for the *dark* set-up solely allowing autoxidation, were placed next to the corresponding clear vials with an extra coverage against light incidence. At each time point, for each analysis type, two clear and two amber vials were transferred to -80 °C until further analysis, allowing duplo analysis for each prepared suspension. For the determination of the fatty acid profile, the PV and the pigment content, the samples were freeze-dried under dark conditions before analysis (Alpha 1-4 LDplus, Christ, Osterode am Harz, Germany).

4.2.3. ANALYSES

4.2.3.1. LIPID EXTRACTION

Prior to the analysis of both the fatty acid profile and the PV, a total lipid extract was needed and obtained from the freeze-dried samples by two serial chloroform:methanol (1:1) extractions according to the method of Ryckebosch et al. [108] as described in 3.2.2.3.

4.2.3.2. FATTY ACID PROFILE

The n-3 LC-PUFA content as part of the fatty acid profile was determined with GC-FID in the same way as was described in 3.2.2.6. Again, the ratio of n-3 LC-PUFA over C16:0 (unstable over stable) was considered. Since the starting values of the different suspensions were not significantly different, a normalization to a starting value of 1 to improve comparison throughout storage was however not required.

4.2.3.3. PRIMARY OXIDATION PRODUCTS

The PV as a measure for primary oxidation was determined with the adapted FOX method as described in 3.2.2.4. Again, the obtained PV was expressed in meq hydroperoxides/100 g suspension and converted to delta values, indicating the relative increase or decrease compared to the start of the storage experiment (day zero).

4.2.3.4. SECONDARY OXIDATION PRODUCTS

Secondary oxidation products (volatiles) were analyzed semi-quantitatively with HS-SPME Arrow GC-MS according to the method as described in 3.2.2.5. 2-Butenal and 2,4-decadienal were added to the list of oxidation markers for reasons explained in the 'Results and Discussion' section (4.3.2.2.). Standards were acquired from Sigma-Aldrich (Steinheim, Germany) for 2-pentenal, 2,4-heptadienal, 2,6-nonadienal and 2,4-decadienal, and from Roth (Karlsruhe, Germany) for 2-butenal. Volatile compound contents (in µg/100 g suspension) were calculated using calibration plots of the standards spiked in an acidic aqueous suspension and expressed as delta values, indicating the relative increase or decrease compared to day zero.

4.2.3.5. PIGMENTS

The pigment profile (including carotenoids and chlorophylls) was analyzed with HPLC-PDA after an acetone:methanol (7:3) extraction. The method used is described in 3.2.2.7. On top of the carotenoid standards used for identification and quantification (antheraxanthin, β-carotene, canthaxanthin, diadinoxanthin, diatoxanthin, echinenone, fucoxanthin, lutein, lycopene, neoxanthin, violaxanthin, zeaxanthin), standards of chlorophyll compounds were also considered: chlorophyll a, chlorophyll b, chlorophyll c2, pheophorbide a, pheophytin a (all from DHI, Horsholm, Denmark). Just like in the previous chapter, diatoxanthin could not be quantified throughout storage for the *Phaeodactylum* systems, because of an overlapping peak of chlorophyll breakdown product after processing. Therefore, regarding the carotenoids, fucoxanthin (as major carotenoid) and β-carotene (as minor carotenoid) were further focused upon.

4.2.4. STATISTICAL ANALYSIS

Results of all analyses were statistically evaluated for the impact of storage time using ANOVA with a significance level of 0.05 and, in case of significance, a post hoc Tukey test to determine the moment at which a significant time effect occurred. For both the n-3 LC-PUFA/C16:0 ratio and the pigment content, an additional one-way ANOVA was performed at each time point to verify the impact of storage condition (dark/light). Furthermore, the interaction effect of storage time and storage condition was investigated in a two-way ANOVA. Due to the nature of lipid oxidation, in which oxidation products can increase or decrease, these additional analyses were not relevant for PV or volatile compounds. For all analyses, JMP Pro 15.1.0 software (SAS Institute Inc., Cary, USA) was used.

4.3. RESULTS AND DISCUSSION

4.3.1. N-3 LC-PUFA EVOLUTION

The evolution of n-3 LC-PUFA during storage is shown in terms of the ratio of n-3 LC-PUFA over C16:0 as a function of storage time in Figure 4.2. It is clear that the *Phaeodactylum* enriched suspensions showed a different trend than the *Schizochytrium* enriched suspensions, and that this trend was not influenced by the (dark/light) storage condition. The ratio of both *Phaeodactylum* enriched suspensions stored in either dark or illuminated conditions remained fairly stable throughout storage. Although significant time effects were observed with p-values of 0.021 and 0.049 for respectively dark and light storage, the *post hoc* Tukey test did not show a clear time point from which the ratio varied significantly. Evaluating the effect of storage condition at each time point, no significant differences were observed, with the exception of the last time point where the ratio for the illuminated samples was (only marginally) significantly lower ($p = 0.048$). This indicated that illumination did not compromise the stability of n-3 LC-PUFA throughout storage. A clear instability, on the other hand, was observed for the *Schizochytrium* enriched suspensions, where the n-3 LC-PUFA/C16:0 ratio decreased from 1.5 to 0.12 (under dark storage) or 0.02 (under light storage) after 28 days of storage at 37 °C, indicating major n-3 LC-PUFA degradation. This decrease was significant after three days for the illuminated suspensions and after seven days for the dark stored ones, which indicated an impact of illumination. Evaluating the impact of storage condition for each time point, the n-3 LC-PUFA/C16:0 content was significantly lower for the illuminated samples after 3, 7, 14 and 28 days of storage. The clearly decreasing trend was thus comparable for both light and dark *Schizochytrium* enriched suspensions, but fell somewhat earlier for the illuminated ones. Regardless of storage condition, this degradation (of at least 90%) was not desired as it nullified the intended n-3 LC-PUFA enrichment. As described by Cheung et al. [215] a decrease in n-3 LC-PUFA content can be caused by either physical losses, lipid oxidation or thermal degradation. Since the ratio of n-3 LC-PUFA over C16:0 was considered, physical losses could be excluded as an explanation. Also thermal degradation should be of minor relevance as the suspensions were not thermally processed and the storage temperature of 37 °C was lower than the high temperatures at which thermal degradation typically occurs [216], leaving lipid oxidation as the most plausible cause for n-3 LC-PUFA degradation in the *Schizochytrium* enriched suspensions. The fact that no significant decrease was observed for the *Phaeodactylum* enriched suspensions under both storage

conditions, is positive for the potential of developing n-3 LC-PUFA rich food products with *Phaeodactylum*, stored in both dark and transparent containers. However, although the occurrence of n-3 LC-PUFA degradation was declarative for lipid oxidation in the previous chapter, its absence for the *Phaeodactylum* systems in the current chapter is still no guarantee for the absence of lipid oxidation, as following up the fatty acid profile alone is still considered as an insensitive method to assess oxidative stability [223] and small amounts of secondary lipid oxidation products can already cause quality problems [7]. Lipid oxidation products are therefore further considered in the next section.

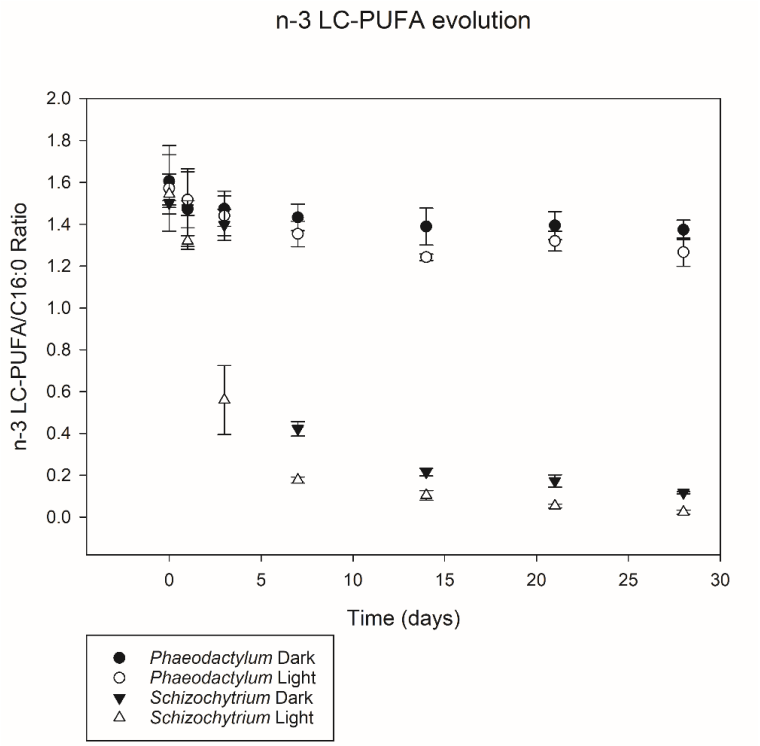


Figure 4.2: Evolution of n-3 LC-PUFA/C16:0 content (mean ± SD, n = 2 x 2) during 28 days of storage at 37 °C under both dark (black symbols) and light (2,600 lux) (white symbols) storage conditions for both *Phaeodactylum* (circles) and *Schizochytrium* (triangles) enriched suspensions.

4.3.2. LIPID OXIDATION PRODUCTS

4.3.2.1. PRIMARY OXIDATION

Figure 4.3 shows the results of the primary oxidation. Again, a clearly different trend could be observed for the *Phaeodactylum* enriched suspensions on the one hand and the *Schizochytrium* enriched suspensions on the other hand, whereas both storage conditions showed similar trends for a certain microalga. For the *Phaeodactylum* enriched suspensions, both dark and light storage showed a significant increase in PV throughout storage (respective p-values of 0.001 and 0.027). With ΔPV values of 0.023 (± 0.004) meq hydroperoxides/100 g suspension (dark) and 0.014 (± 0.009) meq hydroperoxides/100 g suspension (light) after 28 days of storage at 37 °C, this increase was only slight and comparable to former research where *Phaeodactylum* enriched systems were described as being oxidatively stable [110]. Together with the fact that no significant decrease was observed, this indicated that the *Phaeodactylum* enriched suspensions were still in the early stage of lipid oxidation, and this for both dark and light stored samples. Illumination therefore did not seem to promote the formation of hydroperoxides. For the *Schizochytrium* enriched suspensions, a significant decrease following a significant increase could be observed. This revealed strong lipid oxidation as was expected based on former research [110], and as was premised for the current research considering *Schizochytrium* enriched suspensions as a positive control for lipid oxidation to estimate the extent of the possible photo-oxidation for *Phaeodactylum* enriched suspensions. Regarding storage condition, the primary oxidation peak (increase + decrease) occurred significantly earlier for the illuminated *Schizochytrium* samples, with the highest ΔPV value being observed at 3 days of storage for the light stored samples and at 7 days for the dark stored samples. The results of primary lipid oxidation were in line with the results of n-3 LC-PUFA evolution: the appearance of a clear primary oxidation peak for the *Schizochytrium* samples confirmed that the strong n-3 LC-PUFA degradation was due to lipid oxidation, and regarding the impact of illumination, both primary oxidation and n-3 LC-PUFA evolution showed no impact for *Phaeodactylum* enriched suspensions and a small impact (i.e. a similar but earlier trend) for *Schizochytrium* enriched suspensions.

4.3.2.2. SECONDARY OXIDATION

To assess secondary oxidation, several volatile compounds were analyzed, of which Figure 4.4 shows the evolution throughout storage. Depending on the compound considered, different observations could be made.

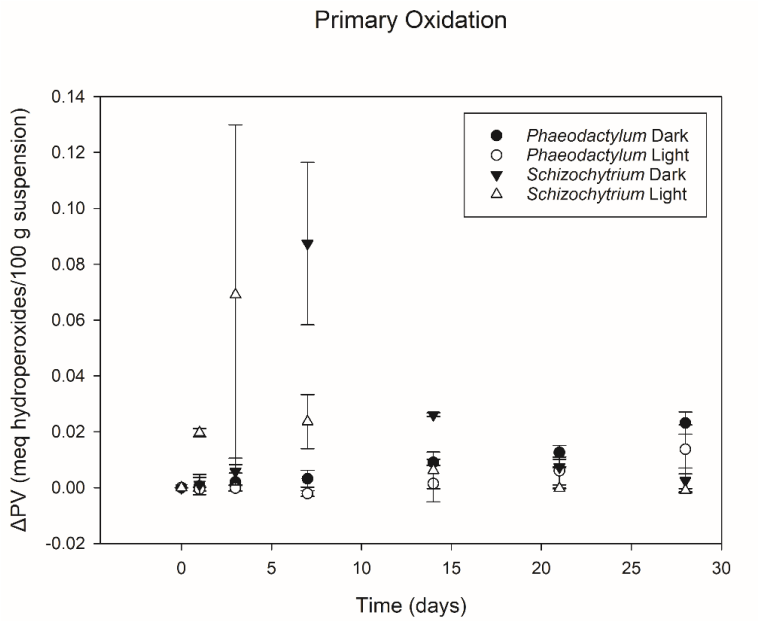


Figure 4.3: Primary oxidation during 28 days of storage at 37 °C under both dark (black symbols) and light (2,600 lux) (white symbols) storage conditions for both *Phaeodactylum* (circles) and *Schizochytrium* (triangles) enriched suspensions. The PV (mean ± SD, n = 2 x 2) was expressed as delta values in meq hydroperoxides/100 g suspension, representing the relative increase or decrease compared to day zero.

First of all, for 2-pentenal, 2,4-heptadienal and 2,6-nonadienal, three compounds that have been reported as specific for n-3 LC-PUFA oxidation [7,163,214], it was clear that for the *Schizochytrium* enriched suspensions these volatile compounds increased significantly for both storage conditions (p-values < 0.001). This confirmed the results of the primary oxidation, which indicated strong lipid oxidation. Also, it could be observed that the amount of these volatiles rose sooner to higher values for the illuminated *Schizochytrium* samples, which was in line with the earlier findings of n-3 LC-PUFA evolution and primary oxidation. Although the increase of these volatile compounds was also significant for the *Phaeodactylum* enriched suspensions (p-values < 0.001), with the exception of 2,6-nonadienal for the light stored samples (p-value = 0,120), it was clearly lower than the increase observed for the *Schizochytrium* enriched suspensions, and this for both storage conditions. This finding was also in line with the former primary oxidation results showing a significant but small increase for *Phaeodactylum* samples.

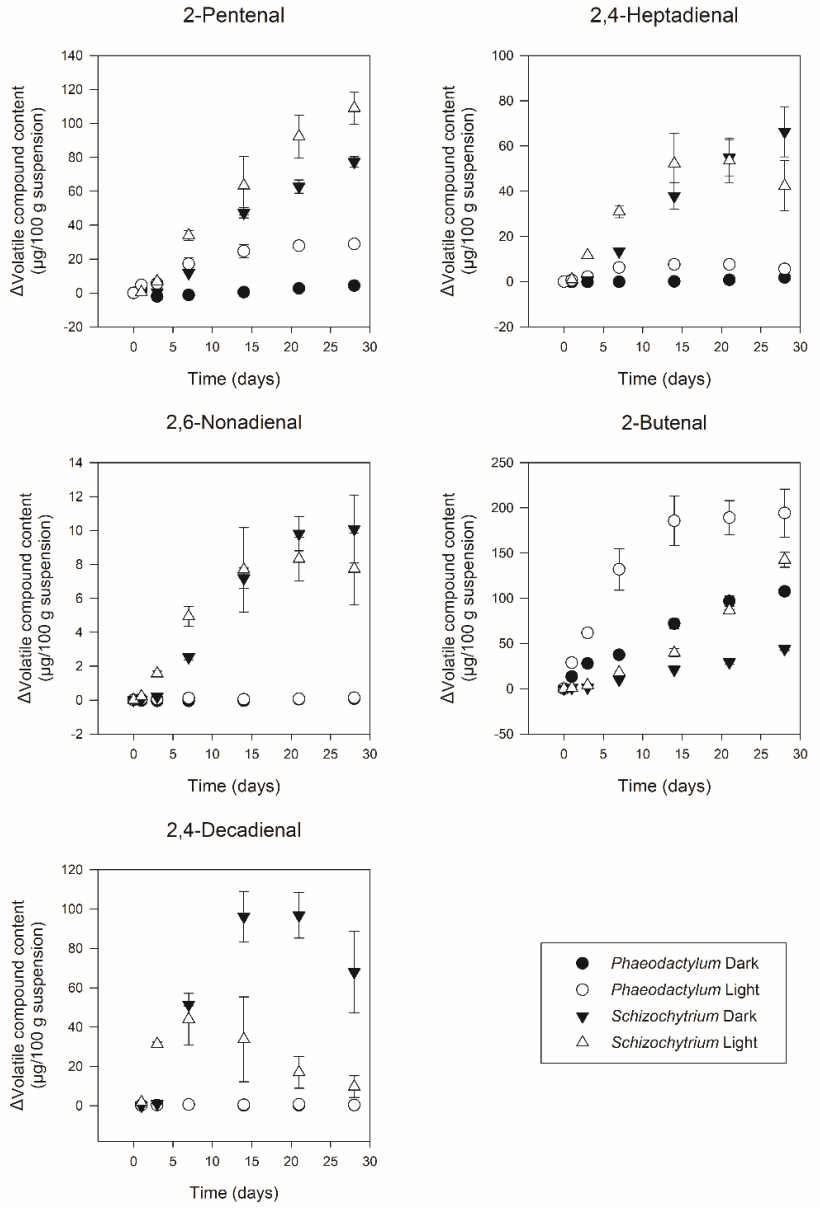


Figure 4.4: Secondary oxidation during 28 days of storage at 37 °C under both dark (black symbols) and light (2,600 lux) (white symbols) storage conditions for both *Phaeodactylum* (circles) and *Schizochytrium* (triangles) enriched suspensions. The content of volatile compounds originating from lipid oxidation [2-pentenal, 2,4-heptadienal, 2,6-nonadienal, 2-butenal and 2,4-decadienal] was expressed as delta values in µg/100 g suspension (mean ± SD, n = 2 x 2), representing the relative increase or decrease compared to day zero.

However, while no impact of illumination was observed for the *Phaeodactylum* enriched suspensions in terms of n-3 LC-PUFA evolution and primary oxidation, both 2-pentenal and 2,4-heptadienal showed a more pronounced increase for the illuminated samples. Moreover, when the evolution of 2-butenal is considered, the illuminated *Phaeodactylum* enriched suspensions showed a significant increase that was clearly higher than for the other suspensions. 2-Butenal can be formed as a secondary oxidation product from n-3 fatty acid specific hydroperoxides (the 17-hydroperoxide for EPA and the 19-hydroperoxide for DHA) that are only formed under direct addition of singlet oxygen and thus under a photosensitized mechanism of oxidation [161]. Therefore these findings suggested that photo-oxidation (under the influence of photosensitizers) was taking place to a certain extent. However, 2-butenal can also be formed as a further oxidation product of 2,4-decadienal[227,228], which is a secondary oxidation product on its own. Although different statements can be found about 2,4-decadienal being either an n-3 or an n-6 fatty acid specific oxidation product[161,163,229], its evolution is therefore also shown in Figure 4.4. The fact that a significant decrease after a significant increase was observed for the *Schizochytrium* enriched suspensions (that again occurred sooner for the illuminated samples), might explain the significant increase in 2-butenal for the *Schizochytrium* samples despite the absence of chlorophyll as a photosensitizer.

4.3.3. OXIDATIVE STABILITY

Considering the results of both primary and secondary oxidation, it is clear that the biggest observed difference in oxidative stability was between the *Schizochytrium* enriched suspensions showing strong lipid oxidation and the *Phaeodactylum* enriched suspensions that were only in an early stage of lipid oxidation. This was in line with former research investigating this difference in terms of autoxidation [110]. Photo-oxidation on the other hand did not seem to play a major role, as illumination only gave rise to rather small differences. The clearest impact of illumination was visible for the already strongly oxidizing *Schizochytrium* samples, in which illumination led to an earlier but similar oxidation. However, to the best of our knowledge, the presence of known photosensitizers has not been reported in *Schizochytrium* biomass, which makes it unlikely that photo-oxidation would be the reason for this. Radiation heat accelerating the already occurring autoxidation processes might be thought of as a possible explanation. The impact of illumination seemed to be even smaller for the *Phaeodactylum* enriched suspensions as it was only visible for certain secondary oxidation markers. The fact that both 2-pentenal and 2,4-heptadienal showed a more pronounced

increase for the illuminated samples might be due to a light induced breakdown of (the low amounts of) hydroperoxides as reported by Schaich et al. [137]. These increases were however still smaller than the observed increases for *Schizochytrium* samples, suggesting only a minor effect. The only indication for the appearance of photo-oxidation in the illuminated *Phaeodactylum* samples was the significant increase for 2-butenal that was clearly higher compared to the other suspensions. The fact that this was not supported by the appearance of a primary oxidation peak indicating the formation of hydroperoxides prior to the formation of 2-butenal, rose questions about the possibility of having missed an early primary oxidation peak in the beginning of the storage experiment. Therefore, the experimental set-up as described above, was repeated for *Phaeodactylum* with a shorter storage experiment having analysis time points already after 0, 2, 4, 8, 16, 24 and 48 hours of storage. The ΔPV value after 1 day for the long storage experiment was similar to the value after 24 hours for the short storage experiment, making comparison valid. However, high amounts of hydroperoxides were also not observed for the shorter time points (data not shown). Therefore it can be stated that photo-oxidation did not cause major oxidative instabilities for the *Phaeodactylum* enriched suspensions on top of the low degree of autoxidation. This is positive for the enrichment potential of *Phaeodactylum* as it gives the opportunity to develop oxidatively stable, n-3 LC-PUFA rich products also in transparent containers.

Comparison with former photo-oxidation research is difficult. The amount of research is limited and the research that is available is very diverse in terms of systems (emulsions [201], oils [204], dairy [206] and solid food products [205,230]), light intensities (varying between 620 lux and 5,340 lux [231] whether or not in the presence of a dark stored control), storage times (hours [232], days [205], weeks [207,233] or even more than a year [234]), storage temperatures (varying between 2 °C [231] and 40 °C [205]), sample storage amounts (varying between 1 mL [201] and 60 g [235] whether or not in closed containers) and analysis methods (in terms of primary and/or secondary oxidation). To the best of our knowledge, photo-oxidation of similar systems as the ones in the current research (i.e. aqueous algal suspensions) was not investigated before. Although the type of system might be determinative for the oxidation results and therefore declarative for possible differences, the research considering n-3 fatty acids or also implementing both dark and light stored samples was touched upon. Oh et al. [205] found that light exposure accelerated lipid oxidation in dried laver (*Porphyra*), a macroalga containing EPA. Although no secondary oxidation products were measured, this effect of

illumination was, in contrast to the current research, visible for both primary oxidation results and EPA content. The appearance of photo-oxidation under illumination was also confirmed in dried, EPA containing macroalgae (*Porphyra yezoensis*) by Sugawara et al. [208] by the presence of non-conjugated hydroperoxide isomers. Studies on O/W emulsions, where n-3 LC-PUFA rich oils were used, did consider both primary and secondary oxidation products, for which higher values could be observed for both analytes in the presence of light and photosensitizers [201,202]. The same could be stated for light exposure in a storage experiment of both crude and refined n-3 LC-PUFA rich fish liver oil [207]. Furthermore, while these effects were all supplementary on an already (to a smaller or larger extent) appearing oxidation, a clear effect of illumination could be observed in the study of Rahmani & Csallany [231] where the presence of light made the difference between oxidative stability (dark) or not (light) for virgin olive oil naturally rich in photosensitizers. In an attempt to explain the absence of clear photo-oxidation in the current research, the pigment evolution is considered in the next section.

4.3.4. PIGMENTS

Figure 4.5 shows the results of the pigment content throughout storage. Since pigments were completely absent in the *Schizochytrium* biomass, these were only relevant for the *Phaeodactylum* enriched suspensions. As was mentioned in the 'Materials and Methods' section (4.2.3.5.), diatoxanthin was, although being slightly present in the *Phaeodactylum* biomass (see 4.2.1.2.), again not considered in this because of quantification no longer being possible after processing due to an overlapping chlorophyll breakdown product peak.

Concerning the carotenoids, both fucoxanthin and β-carotene were well detected. Regardless of storage condition, they showed a significant decrease throughout storage: from ca. 28 mg/100 g suspension to ca. 4 mg/100 g suspension for fucoxanthin and from ca. 2 mg/100 g suspension to ca. 1 mg/100 g suspensions for β-carotene after 28 days of storage at 37°C. These degradations were comparable to former research investigating dark stored *Phaeodactylum* enriched tomato purees [8], and pointed towards their antioxidative role towards autoxidation. As was explained in the previous chapter, carotenoids can react with peroxyl radicals or scavenge oxygen, which both lead to an inhibition of lipid oxidation [236]. Evaluating the impact of storage condition, the interaction effect between storage time and storage condition was not significant for fucoxanthin (p-value = 0.353) and significant for β-carotene (p-value = 0.030), indicating that

only for β-carotene the time effect was significantly dependent on the storage condition. Further investigating the impact of storage condition at each time point, the only significant differences were observed at 14 days for β-carotene (p-value = 0.027) and at 21 days for both fucoxanthin and β-carotene (p-values of 0.011 and 0.019). Therefore it could be stated that illumination did not have a major impact on the carotenoid content throughout storage, which was in line with the results of primary oxidation and n-3 LC-PUFA evolution.

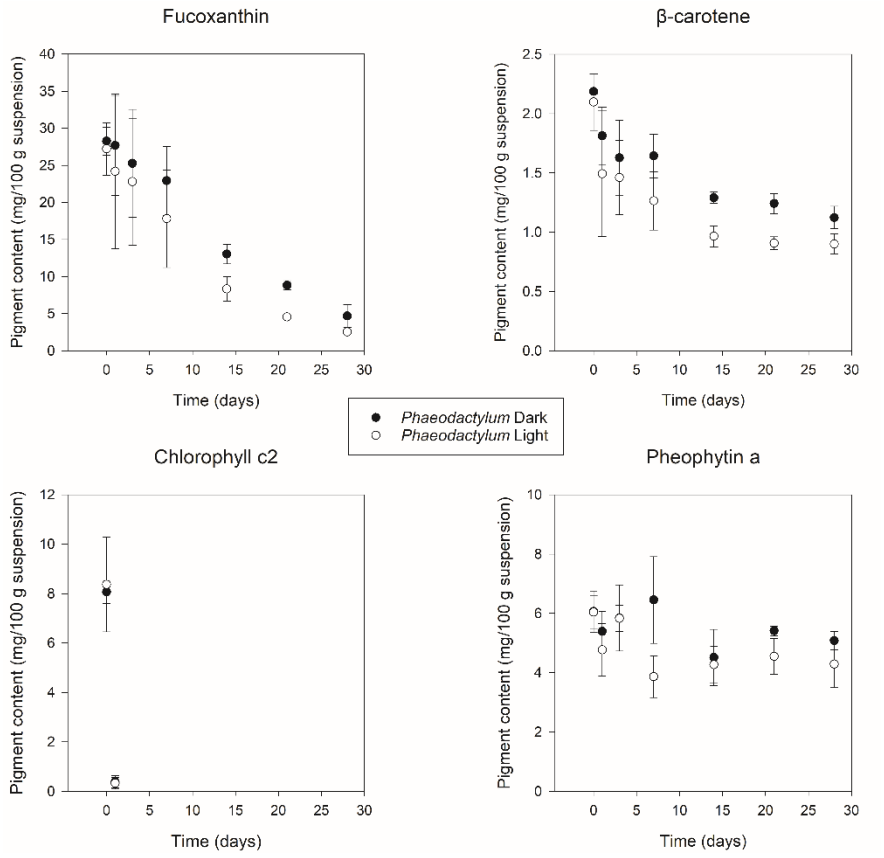


Figure 4.5: Evolution of pigment content (fucoxanthin, β-carotene, chlorophyll c2 and pheophytin a) in mg/100 g suspension (mean ± SD, n = 2 x 2) during 28 days of storage at 37 °C under both dark (black symbols) and light (2,600 lux) (white symbols) storage conditions for the *Phaeodactylum* enriched suspensions. Pigments were absent in the *Schizochytrium* enriched suspensions.

As was mentioned before, carotenoids are known for their quenching ability in preventing photo-oxidation. In contrast to reaction with peroxyl radicals, physical

quenching however does not cause carotenoid degradation [140]. Therefore the fact that no supplemental carotenoid degradation was observed for illuminated samples, does not mean that they did not exert an antioxidative role towards photo-oxidation. On the contrary, the fact that both carotenoids were also present under illuminated conditions throughout storage might be a reason for the absence of clear photo-oxidation in the current research, as they were constantly available as possible quenchers.

In comparison with former research, it is difficult to state whether the observed carotenoid amount could be expected to be sufficient for the prevention of photo-oxidation. This is again due to the diversity in set-up and analysis methods. Nevertheless, β-carotene has repeatedly been reported as being a strong physical quencher [237]. Shimidzu et al. [238] compared the quenching activity of several carotenoids, and observed that the quenching activity of fucoxanthin was approximately ten times lower than the one of β-carotene. In the current research, β-carotene might therefore be equally important for photo-oxidative stability as fucoxanthin despite its clearly lower abundance.

Other pigments in *Phaeodactylum* biomass were chlorophyll a, chlorophyll c2 and pheophytin a. Chlorophyll a was not detected throughout the storage experiment, indicating that there was a complete degradation during the preparation of the suspensions before the start of the storage experiment. This may be explained by the degradation susceptibility of chlorophyll a at low pH [239] such as in the model systems in this study, and a possible enzymatic breakdown by chlorophyllase of which the presence has been reported in *Phaeodactylum* [240]. Chlorophyll c2 was detected at the beginning of the storage experiment, but was also characterized by a fast degradation (Figure 4.5). For both storage conditions, the chlorophyll c2 decreased from an amount of ca. 8 mg/100 g suspension to <1 mg/100 g suspension after one day of storage, and was not detected anymore after three days of storage. Again, illumination did not have a significant impact. Pheophytin a was therefore the only chlorophyll-like pigment that was present throughout storage (Figure 4.5), showing no clear increasing or decreasing trend and, apart from a single time point at 7 days, no significant impact of illumination. Regarding the above lipid oxidation results, the rapid disappearance of chlorophylls might be the reason for the lack of a clear photo-oxidation effect as their well-known photosensitization properties [7] disappear with their absence. However pheophytin a has also been stated as being a potent photosensitizer [241]. Although the actual research on this is limited, Rahmani & Csallany [231] reported that pheophytin a, present at a concentration of 8.54 ppm as the only plausible

photosensitizer, functioned as a photosensitizer causing rapid lipid oxidation in olive oil in the presence of light. Therefore, it could not be stated that photosensitizing potential was completely absent in the *Phaeodactylum* enriched suspensions. The above hypothesis of carotenoids being sufficiently present as possible quenchers throughout storage, to explain the absence of clear photo-oxidation, therefore still makes sense.

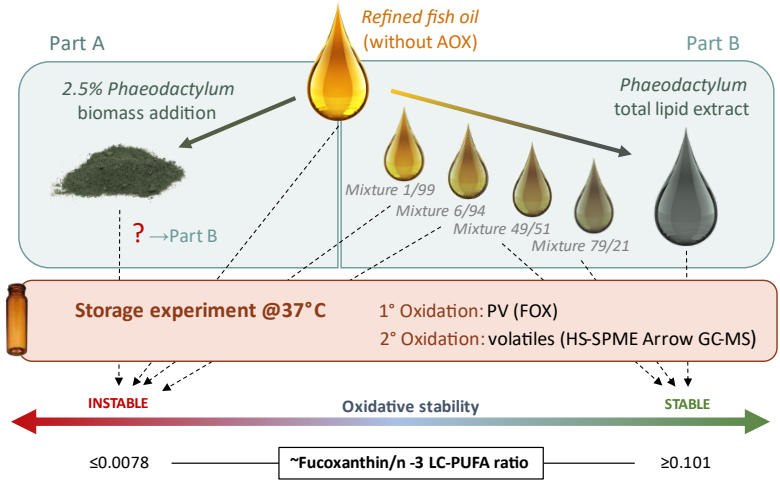
4.3.5. Transparency

From the results of both primary and secondary oxidation, it was clear that illumination did not cause major supplemental lipid oxidation due to photo-oxidation in *Phaeodactylum* enriched suspensions. Certainly not if these were compared with the strong lipid oxidation that was observed under dark as well as light storage conditions for *Schizochytrium* enriched suspensions. Only the increase of 2-butenal during storage suggested a low appearance of photo-oxidation. The reason for this minor effect could be due to fucoxanthin and β-carotene being constantly sufficiently present throughout storage as possible quenchers and/or due to a fast chlorophyll breakdown leaving pheophytin a as the only possible photosensitizer of which the photosensitizing properties are not extensively described. Apart from that, the transparency of the samples could also play an important role. Looking at the enriched suspensions, this is expected to be rather low, certainly if it would be compared to the nanoemulsions in former photo-oxidation research of which a transparent appearance is a desired characteristic [201]. Uluata et al. [203] reported that for riboflavin enriched fish oil-in-water emulsions, an increase in transparency (caused by a decrease in droplet size) led to a higher degree of photo-oxidation, and Rahmani & Csallany [231] also clearly showed that an increase in light intensity (considering 0; 620; 2,710 and 5,340 lux) led to stronger lipid oxidation in virgin olive oil. Therefore the observed low transparency of the suspensions in the current research, might cause photo-oxidation to only have a minor impact, as hence a lower volume average light intensity is expected to be perceived by the whole sample.

4.4. CONCLUSIONS

Photo-oxidation did not cause oxidative instability for *Phaeodactylum* enriched aqueous model systems. Compared with the strong autoxidation in *Schizochytrium* enriched systems, the supplemental oxidation upon illumination was minor at most. Three different factors may underlie this limited impact: the sufficient presence of fucoxanthin and β-carotene as possible quenchers, the limited amount of photosensitization by fast chlorophyll to pheophytin breakdown and the low transparency. Either way, the finding that illumination did not cause a major supplemental lipid oxidation is positive for the further development of photoautotrophic microalgae (and in particular *Phaeodactylum*) as an alternative n-3 LC-PUFA source in food products (specifically aqueous food products with a low pH), since enrichment also leads to oxidatively stable products in the presence of light, as would be the case for products stored in transparent containers.

CHAPTER 5 - THE POTENTIAL OF *PHAEODACTYLUM* AS A NATURAL SOURCE OF ANTIOXIDANTS FOR FISH OIL STABILIZATION



Adapted from: Demets, R., Van Broekhoven, S., Gheysen, L., Van Loey, A., & Foubert, I. (2022). The Potential of *Phaeodactylum* as a Natural Source of Antioxidants for Fish Oil Stabilization. *Foods*, 11 (10), 1461.

5.1. INTRODUCTION

Phaeodactylum was shown to give rise to enriched aqueous model systems that are oxidatively stable in both dark and light storage conditions, which was attributed to the sufficient abundance of endogenous carotenoids (Chapters 3 and 4). Therefore, the question rises whether these carotenoids would also be capable of stabilizing other lipids on top of the microalgal n-3 LC-PUFA and in that way be used as novel (natural) antioxidants. In other words, the new line of questioning introduced in the current chapter is on how capable these carotenoids, or the microalgae containing them, are in stabilizing n-3 LC-PUFA in general. To investigate this, a research shift was made from aqueous model systems towards bulk oil systems, more specifically with n-3 LC-PUFA rich fish oil as a base.

As was mentioned in the literature review (Part I), fish oil is worldwide an important source of n-3 LC-PUFA for both food (supplements or food enrichment) and feed (aquaculture, cattle and poultry) applications (Chapter 1). It is however troubled by a high lipid oxidation susceptibility, as was specifically addressed in Chapter 2 (2.3.1.1.). In avoiding the use of strong synthetic antioxidants due to their potential negative health effects, the search for natural antioxidants able to inhibit fish oil oxidation has received a lot of attention [16]. Apart from tocopherols and rosemary extracts being the most frequently used natural antioxidants, a broad range of plant sources containing polyphenolic compounds has been explored, as reviewed by Hrebień-Filisińska [16]. Studies on marine natural antioxidant sources for the inhibition of fish oil oxidation on the other hand have been rather limited with the major attention attributed to macroalgal extracts [242–247]. Microalgae have only scarcely been addressed with Golmakani et al. [248] showing the potential of an *Arthrospira platensis* extract in stabilizing kilka oil.

Research on microalgae as a source of antioxidants in bulk oil has up to now been focused on vegetable oils. Moreover, apart from Lee et al. [249] showing inhibition of soybean oil oxidation by addition of *Nannochloropsis* biomass, all studies in this regard focused on non-'n-3 LC-PUFA rich' species of which either microalgal biomass or a microalgal extract is added. Studies with microalgal biomass addition encompassed the addition of either *Chlorella* or *Arthrospira* to olive oil and all reported an enhanced oxidative stability attributable to the gradual release of antioxidants [250–254]. Studies with microalgal extract addition were more diverse in terms of species (*Haematococcus* [255,256], *Scenedesmus* [257], *Chlorella* [258] and *Anabaena* [259]), extraction method (solvent extraction with methanol, hexane or

acetone or supercritical fluid extraction with CO_2) and vegetable oil (soybean, sunflower, olive or others) but also reported an increase in oxidative stability explainable by the presence of carotenoids. Only Gouveia et al. [258], investigating the addition of a *Chlorella* supercritical CO_2 extract in soybean oil, did not observe an antioxidative effect.

Based on these studies it is clear that microalgae show potential for the inhibition of bulk oil oxidation. The aim of the research in the current chapter is to investigate whether this also holds for fish oil as an n-3 LC-PUFA rich and therefore oxidatively more susceptible oil, using n-3 LC-PUFA rich microalgae known to be stable towards n-3 LC-PUFA oxidation, like *Phaeodactylum*, as was shown in the previous chapters. Foo et al. [260] already suggested the antioxidative capacities of *Phaeodactylum*, although only based on direct antioxidant assays. The experimental set-up was twofold. In a first part, the antioxidative effect of supplementing fish oil with *Phaeodactylum* sp. biomass was investigated by analyzing both primary and secondary oxidation products throughout an accelerated storage experiment (3 weeks at 37 °C). In a second part, an elucidating approach was followed to get more insight into the required amount of microalgal antioxidants needed to inhibit n-3 LC-PUFA oxidation and to explain the results of the first experimental set-up. For this, a total lipid extract (chloroform:methanol 1:1) of the *Phaeodactylum* biomass was made and mixed with fish oil in different proportions to obtain six different systems (containing 100, 79, 49, 6, 1 and 0 weight% of the *Phaeodactylum* total lipid extract). Again, the oxidative stability was evaluated by analyzing primary and secondary oxidation products throughout an accelerated storage experiment (10 weeks at 37 °C). Other than the first experimental set-up, the second was not aimed at a practical application. Rather than investigating the potential of the addition of *Phaeodactylum* total lipid extract as an antioxidant, the objective was to clarify the n-3 LC-PUFA oxidation inhibition capacity of the antioxidative compounds present in this extract from a scientific point of view.

5.2. MATERIALS AND METHODS

5.2.1. MATERIALS

5.2.1.1. RAW MATERIALS

Freeze-dried *Phaeodactylum* sp. biomass was obtained from Archimede Ricerche (Camporosso, Italy). The n-3 LC-PUFA content was determined as 20.0 (±0.3) mg/g dry biomass and the carotenoid profile consisted of 5.3 (±0.5) mg fucoxanthin, 0.6 (±0.1) mg diatoxanthin and 0.48 (±0.02) mg β-carotene per g dry biomass. Fresh fish oil, refined and without added antioxidants, was kindly provided by INVE (Baasrode, Belgium) and had an n-3 LC-PUFA content of 243 (±1) mg/g oil. No carotenoids were detected. PV (as determined by INVE) was 4.13 meq hydroperoxides/kg oil. N-3 LC-PUFA and carotenoid content were determined using the analyses as described below. Both *Phaeodactylum* sp. biomass and fish oil were stored at -80 °C until further use.

5.2.1.2. CHEMICALS

The used chemicals are among those that were already described in a previous chapter (3.2.2.2.).

5.2.2. EXPERIMENTAL SET-UP

The experimental set-up consisted of two parts of which Figure 5.1 shows a schematic overview. 'Part A' investigated the oxidative stability of two systems: fish oil with and without added *Phaeodactylum* biomass. For 'Part B' six different systems obtained after mixing a *Phaeodactylum* total lipid extract with fish oil in different proportions were considered. Details of both experimental set-ups are discussed below.

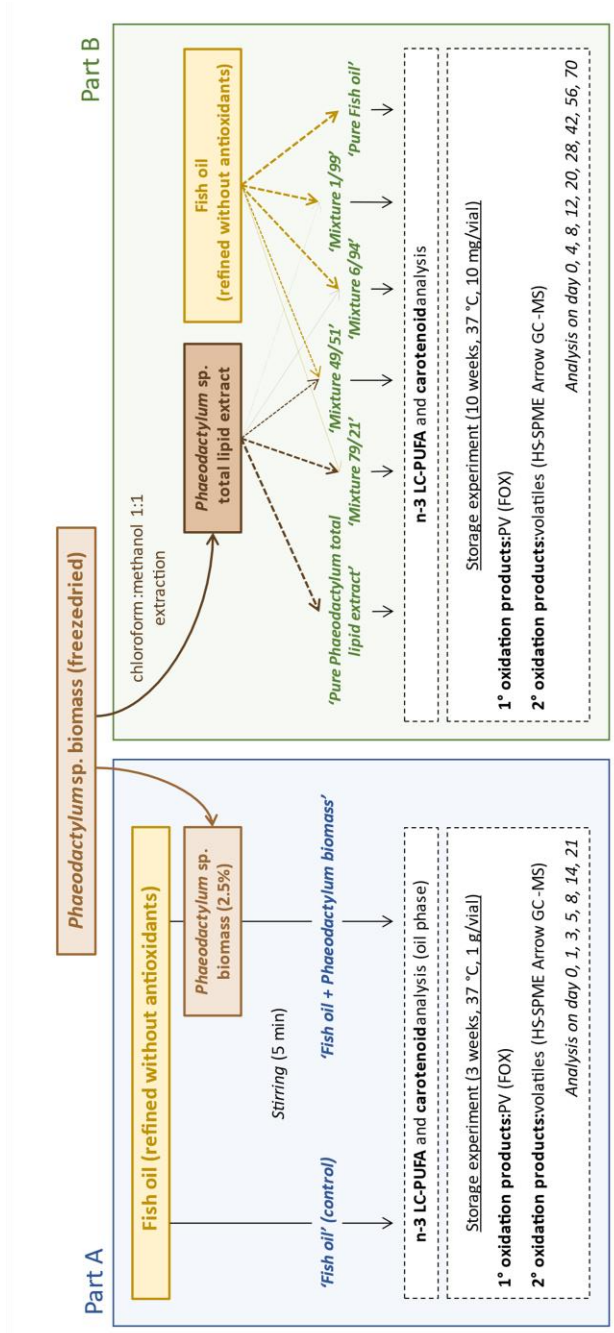


Figure 5.1: Experimental set-up

5.2.2.1. PART A

The fish oil was mixed with 2.5% (w/w) *Phaeodactylum* biomass and stirred for 5 min (room temperature, 300 rpm, IKA RO10 stirrer) to obtain the '*Fish oil + Phaeodactylum biomass*' system. The concentration was chosen in line with former research adding microalgal biomass to bulk oil [249–254]. To get a correct impression of the influence of *Phaeodactylum* biomass addition on the oxidative stability, also a '*Fish oil*' (control) system was considered, obtained by stirring pure fish oil for 5 min without *Phaeodactylum* biomass addition. The systems were characterized in terms of n-3 LC-PUFA content and carotenoid profile according to the analyses described below. Analyses of the '*Fish oil + Phaeodactylum biomass*' system were performed on the oil phase obtained after sedimentation of the solid parts by a centrifugation step of 5 min at 200 g (SL 16 Centrifuge, Thermo Scientific). To determine the oxidative stability, a storage experiment of 3 weeks was set up. Both systems were transferred to amber screw-capped 20 mL vials in quantities of 1 g/vial, and while doing so, the systems were stirred with a magnetic stirrer to ensure a homogenous distribution. Vials were incubated (Memmert IN160 incubator, Hettich) at 37 °C, a temperature that is assumed to accelerate the oxidation without changing its mechanisms [148], and transferred to -80 °C until further analysis after seven different time points (day 0, 1, 3, 5, 8, 14, 21). For each time point, primary (PV) and secondary (volatile compounds) oxidation products were determined in duplicate according to the methods described below, starting from independent vials. For the PV analysis of the '*Fish oil + Phaeodactylum biomass*' system, the oil phase as separated by centrifugation was used again. A storage experiment analyzing the evolution of both primary and secondary oxidation products throughout storage was opted for to get a realistic indication of the oxidative stability and therefore the underlying antioxidant potential, rather than using quick antioxidant assays which only give a method dependent indication [190] like was touched upon in 2.2.4.

5.2.2.2. PART B

i. *PHAEODACTYLUM TOTAL LIPID EXTRACT*

The second part of the experimental set-up started with obtaining a total lipid extract from the *Phaeodactylum* biomass. For this, a large scale chloroform:methanol (1:1) extraction was used based on the method described by Ryckebosch et al. [108]. The following steps were performed in eightfold: 22.5 mL of the solvent was added to 3 g *Phaeodactylum* biomass and stirred for 90 min, after centrifugation (10 min, 750 g) the solvent phase was separated and the same

procedure was repeated, both combined solvent phases were washed with 11.25 mL water and after vigorous shaking (2 min), centrifugation (10 min, 750 g) and removal of the upper water phase, the solvent phase was dried over sodium sulfate using a Whatman 1 filter that was washed with chloroform:methanol (1:1) afterwards. All eight filtered extracts were pooled to obtain one homogenous extract and the solvent was removed by rotary evaporation under reduced pressure at 40 °C.

ii. *PREPARATION AND CHARACTERIZATION OF THE SYSTEMS*

Starting from the *Phaeodactylum* total lipid extract and the fish oil, six different systems were prepared by mixing them in different proportions. They were denoted as '*Pure Phaeodactylum total lipid extract*', '*Mixture 79/21*', '*Mixture 49/51*', '*Mixture 6/94*', '*Mixture 1/99*' and '*Pure Fish* oil'. Table 5.1 shows an overview of the ratios used (in weight%). These ratios were chosen in such a way that different orders of magnitude of the amount of carotenoids relative to the amount of n-3 LC-PUFA could be evaluated. To ensure homogenous mixing, both phases were dissolved in chloroform:methanol (1:1) before combining them. All systems were characterized in terms of n-3 LC-PUFA content and carotenoid profile. For this, part of the mixture was taken aside, flushed dry with nitrogen, and analyzed in triplicate according to the methods described below.

Table 5.1: Ratios (in weight%) of the *Phaeodactylum* total lipid extract and Fish oil used for preparing the different systems of Part B of the experimental set-up

System	***Phaeodactylum* total lipid extract** (weight%)	**Fish oil** (weight%)
Pure Phaeodactylum total lipid extract	100.0	0.0
Mixture 79/21	78.6	21.4
Mixture 49/51	49.2	50.8
Mixture 6/94	6.4	93.6
Mixture 1/99	0.7	99.3
Pure Fish oil	0.0	100.0

iii. *OXIDATIVE STABILITY (STORAGE EXPERIMENT)*

The oxidative stability of the different systems was followed throughout a storage experiment of 10 weeks at 37 °C, with in triplicate analysis of primary and secondary oxidation products (according to the methods described below) on the following time points: day 0, 4, 8, 12, 20, 28, 42, 56 and 70. The systems that already showed clear oxidation in the first 4 weeks were only analyzed until day

28. The systems were incubated in quantities of 10 mg in independent amber screw-capped 20 mL vials, obtained after distributing the different mixed systems in solvent and dry flushing with nitrogen. After incubation and until analysis, the vials were transferred to -80 °C.

5.2.3. ANALYSES

5.2.3.1. N-3 LC-PUFA CONTENT (CHARACTERIZATION)

The n-3 LC-PUFA content (as part of the fatty acid profile) was determined with GC-FID after methyl transesterification according to the method as described in 3.2.2.6. To enable absolute quantification, a known amount of lauric acid (C12:0) (Nu-Check Prep, Elysian, USA) was added as an internal standard before derivatization.

5.2.3.2. CAROTENOIDS (CHARACTERIZATION)

Carotenoids were determined with HPLC-PDA according to the method as described in 3.2.2.7. with the difference that no extraction was needed. Instead, prior to HPLC analysis, a 10 mg lipid sample was dissolved in 10 mL acetone:methanol (7:3), filtered through a PVDF syringe filter (4 mm, 0.2 µm) and diluted 1/3.

5.2.3.3. PRIMARY OXIDATION PRODUCTS (STORAGE)

The amount of primary oxidation products or PV, was again analyzed with the adapted FOX method as described in 3.2.2.4. The results were expressed as delta values, representing the relative increase or decrease compared to day zero, to focus on the trend of oxidation during storage.

5.2.3.4. SECONDARY OXIDATION PRODUCTS (STORAGE)

The degree of secondary oxidation was determined by analysis of specific volatile n-3 LC-PUFA oxidation markers with HS-SPME Arrow GC-MS according to the set-up as described in 3.2.2.5. Only the compounds with a solid identification for all time points when using the NIST MS Search 2.3 spectral library and of which a standard was available to confirm the retention time, were further reported. These were: 2,4-heptadienal, 2-pentenal and 2-butenal. 2,6-nonadienal and 2,4-decadienal were characterized by a questionable identification due to overlapping compounds in the investigated (lipid) matrices and were therefore not included. Although 2-butenal was approached as an early photo-oxidation marker in the previous chapter (4.3.2.2.), it has also been reported as a further

breakdown product of volatile oxidation products of autoxidation [227,228] explaining why it is nevertheless considered in the current chapter. Calibration plots were again set up by spiking these standards in the matrix of interest. For Part B, hexanal d-12 (CDN Isotopes, J.H. Ritmeester B.V., Nieuwegein, Netherlands) was spiked as an internal standard for each analysis. The data (expressed in µg/mg) were again expressed as delta values, representing the relative increase or decrease compared to day zero, to focus on the oxidation trend.

5.2.4. STATISTICAL ANALYSIS

For the results of both primary and secondary oxidation, the impact of storage time was statistically evaluated for all systems with JMP Pro 15.1.0 software (SAS Institute Inc., Cary, USA). One-way ANOVA with a significance level of 0.05 was used with a post hoc Tukey test in case of significance to determine the time points showing relevant increases or decreases.

5.3. RESULTS AND DISCUSSION

5.3.1. PHAEODACTYLUM BIOMASS ADDITION (PART A)

5.3.1.1. PRIMARY OXIDATION

Figure 5.2 shows the primary oxidation results of the fish oil with and without *Phaeodactylum* biomass addition. Both systems show a significant increase towards high ΔPV values, with the highest ones being observed after 14 days of storage: respectively 250 (±37) and 397 (±8) meq hydroperoxides/kg lipids with and without *Phaeodactylum* biomass addition. The order of magnitude of these values was comparable to former research investigating primary oxidation during storage of bulk fish oil [81,261]. After 21 days, a decrease could be observed. This characteristic 'increase - decrease' pattern of primary oxidation products indicates strong lipid oxidation [7], and this for fish oil as well as for fish oil with 2.5% *Phaeodactylum* biomass addition. Although *Phaeodactylum* biomass addition gave rise to lower ΔPV values for most of the time points (between 8 and 161 meq hydroperoxides/kg lipids lower), it did not inhibit the lipid oxidation given the increase and decrease of primary oxidation products at the same time points. A possible reason for the lower ΔPV values despite the increase and decrease at similar time points, could be found in the polarity increase of hydroperoxidized lipids. As described by Junqueira et al. [262] who investigated molecular organization in hydroperoxidized lipid bilayers, the '-OOH moiety' preferentially locates towards the more polar regions. Given the sampling method of the 'Fish oil + *Phaeodactylum* biomass' system in which the oil phase was separated from the solid *Phaeodactylum* parts by centrifugation prior to PV determination, this could have caused an underestimation of hydroperoxides by an accumulation away from the apolar bulk oil phase towards the *Phaeodactylum* solid parts.

5.3.1.2. SECONDARY OXIDATION

Figure 5.3 shows the evolution of 2,4-heptadienal, 2-pentenal and 2-butenal as a measure for secondary n-3 LC-PUFA oxidation. For both systems these three compounds showed a clear increase of respectively 0.06 µg/mg, 0.05 µg/mg and 0.03 µg/mg throughout storage. The fact that a clearer and significant increase was observed towards the end of the storage experiment (after 14 and 21 days) while the primary oxidation products showed a sooner significant increase (during the first storage week) is logic as these compounds are formed through hydroperoxide decomposition [161,164,214] (Figure 2.2 of Chapter 2 is referred to).

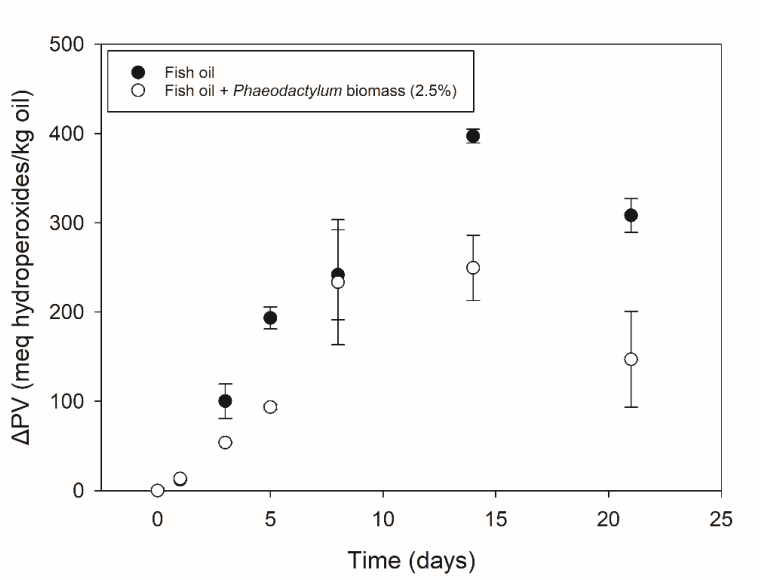


Figure 5.2: Primary oxidation of fish oil with (white symbols) and without (black symbols) 2.5% *Phaeodactylum* biomass addition during 21 days of storage at 37 °C. The PV (mean ± sd, n=2) is expressed as delta values, representing the relative increase or decrease compared to day zero.

Other than for the primary oxidation results, the fish oil systems with *Phaeodactylum* biomass addition (roughly) showed an equally large (but certainly not lower) increase compared to the pure fish oil systems. Apart from making the above mentioned hypothesis of the hydroperoxide underestimation more probable, this again indicates a clear lipid oxidation independent of 2.5% *Phaeodactylum* biomass addition.

5.3.1.3. ANTIOXIDATIVE CAPABILITY

Based on the results of primary and secondary oxidation, it is clear that the *Phaeodactylum* biomass addition did not exert an antioxidative effect. In other words, *Phaeodactylum* biomass was not capable of inhibiting fish oil oxidation. Former studies that added microalgal biomass to bulk oil did however report an enhancement of oxidative stability [249–254]. Nonetheless, it has to be remarked that Lee et al. [249], who investigated the addition of *Nannochloropsis* biomass to soybean oil, based these findings on a Rancimat test operating at temperatures of 120-150 °C far from real storage conditions, while single PV values after 15 h (without further storage) were even shown to be slightly higher (ca. 2 meq hydroperoxides/kg oil) for the oil with added *Nannochloropsis* biomass.

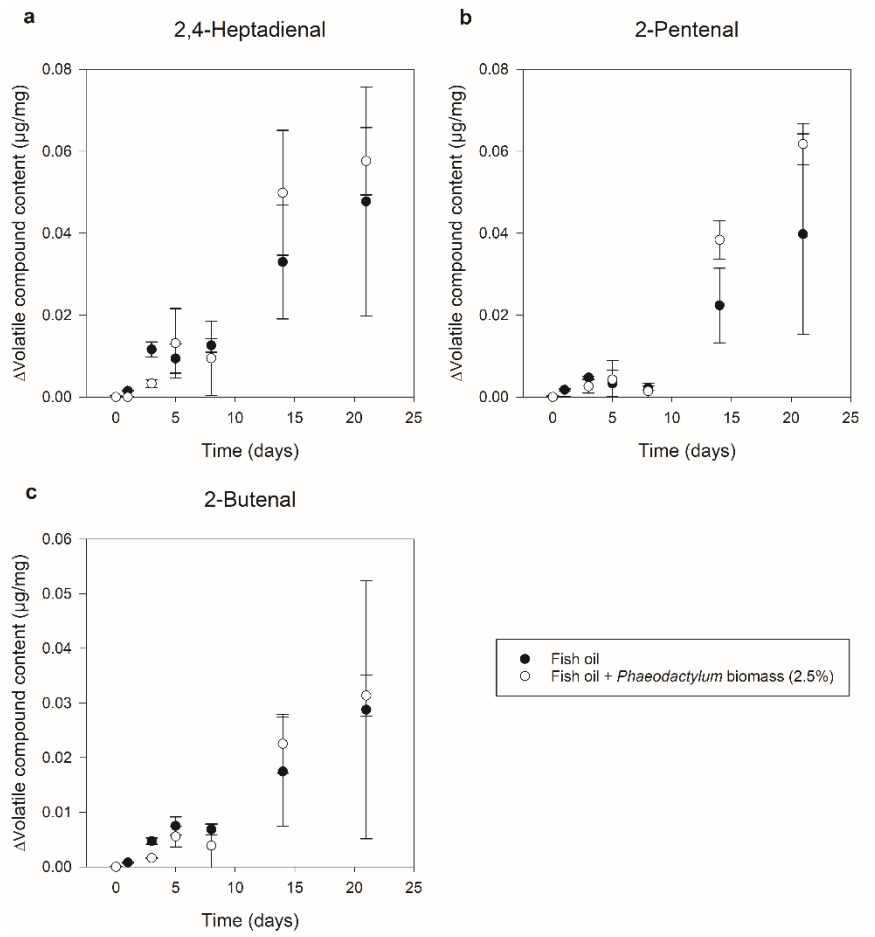


Figure 5.3: Secondary oxidation of fish oil with (white symbols) and without (black symbols) 2.5% *Phaeodactylum* biomass addition during 21 days of storage at 37 °C. The content of volatile compounds originating from lipid oxidation [2,4-Heptadienal (a), 2-Pentenal (b) and 2-Butenal (c)] in µg/mg (mean ± sd, n = 2) is expressed in delta values, representing the relative increase or decrease compared to day zero.

Also the results of the studies of Alavi & Golmakani [250,252] require nuance. Although microalgal biomass addition (in this case *Chlorella* or *Arthrospira*) to virgin olive oil did lead to lower PV values (of ca. 40-100 meq hydroperoxides/kg oil lower), this was only the case for the last time point of the 42 days storage experiment which compared to the earlier time points still showed a clear increase of ca. 50-100 meq hydroperoxides/kg oil and therefore an oxidation onset for oils both with and without microalgal biomass. Moreover it has to be kept in mind that, other than in the current research, systems without

n-3 LC-PUFA were considered and also the methods of analyzing lipid oxidation showed some differences since a storage temperature of 60 °C and a less specific analysis method for secondary oxidation (anisidine value) were used. This was also the case in the study of Morsy et al. [254] which investigated the effect of adding both whole cell *Arthrospira* and *Arthrospira* nanoparticles (after ball-milling) to olive oil, although in this study lower PV values (4-58 meq hydroperoxides/kg oil lower) without a clear oxidation onset were observed for all time points (weekly analysis for 7 weeks) making their statement of *Arthrospira* exerting an antioxidative action more powerful. Since in all of these papers the antioxidative effect is explained by the release of antioxidative compounds from the microalgal biomass into the oil, it raises the question whether this sufficiently occurred in the current research or whether the reason for the absence of oxidation inhibition lies in the fact that no antioxidants were released from the *Phaeodactylum* biomass. With former research and previous chapters suggesting carotenoids as the most important antioxidants in *Phaeodactylum* [8,260,263], their presence in the fish oil after mixing with *Phaeodactylum* biomass was investigated and is discussed in the next section.

5.3.1.4. CAROTENOID CONTENT

Table 5.2 shows the results of the carotenoid characterization of the oil phase of the *Phaeodactylum* biomass enriched fish oil system. It concerns the content of carotenoids after preparing the system (i.e. at the start of the storage experiment). Just like in the *Phaeodactylum* biomass, fucoxanthin was the most abundant carotenoid followed by diatoxanthin and β-carotene. The fact that carotenoids were detected in the oil showed that during stirring a certain carotenoid transfer from the *Phaeodactylum* biomass to the fish oil occurred, as no carotenoids were present in the pure fish oil. Based on the amount of fucoxanthin in the *Phaeodactylum* biomass (5.31 (±0.52) mg/g dry biomass), the amount of fucoxanthin in the oil phase in case of full transfer would have been 133 (±13) µg/g, indicating that ca. 12 (±3)% of the fucoxanthin migrated. Although it could therefore be stated that the absence of antioxidant migration was not the reason for the lack of oxidation inhibition, it is still possible that the amount did not suffice for an antioxidative effect. Limon et al. [257] reported carotenoid contents of ca. 20 µg β-carotene and ca. 6 µg lutein per g oil in their study that did describe an antioxidative effect of *Scenedesmus* extract addition to virgin olive oil. These contents are comparable to the amount of fucoxanthin detected in the current research. Although the differences in set-up regarding raw materials (not containing n-3 LC-PUFA) and analysis methods (Rancimat) could again be

remarked as possible reasons for different findings, the question remains why the microalgal antioxidants in the current research were not capable of keeping the n-3 LC-PUFA rich fish oil oxidatively stable while their role in doing so has been suggested for the n-3 LC-PUFA rich microalgal oil or biomass itself [8,81].

Table 5.2: Oil phase carotenoid content of the 'Fish oil + *Phaeodactylum* biomass' system

Carotenoid	Content (µg/g oil)	Carotenoid/n-3 LC-PUFA ratio (w/w)
Fucoxanthin	17 (±4)	0.00007 (±0.00002)
Diatoxanthin	4.4 (±0.7)	0.000018 (±0.000003)
β-carotene	< LOQ (=5)	< 0.00002

In trying to address this question, apart from only considering the carotenoid content itself, also the amount relative to the amount of n-3 LC-PUFA (the compounds causing oxidative susceptibility) was examined. Table 5.2 shows the ratio of carotenoid to n-3 LC-PUFA content of the oil phase, with the fucoxanthin to n-3 LC-PUFA ratio being 0.00007 (±0.00002). If this is compared with the pure *Phaeodactylum* biomass having a fucoxanthin/n-3 LC-PUFA ratio calculated to be 0.27 (±0.03), the big difference in order of magnitude is clear. Despite antioxidants being defined as compounds that delay or prevent oxidation when present at low concentration compared with those of the oxidizable substrate [244], the observed concentrations in the oil phase may simply be too low. Investigating the amount that would suffice for stabilizing the fish oil could give more insight into the capacity of *Phaeodactylum* as antioxidant and forms the basis of the second part of the experimental set-up. The potential of *Phaeodactylum* biomass addition will be returned to at the end of Part B taking into account its results.

5.3.2. PHAEODACTYLUM TOTAL LIPID EXTRACT – FISH OIL MIXTURES (PART B)

5.3.2.1. CHARACTERIZATION (FUCOXANTHIN/N-3 LC-PUFA RATIO)

Building further on the amount of fucoxanthin relative to the amount of n-3 LC-PUFA, Table 5.3 gives the n-3 LC-PUFA content (in mg/g), fucoxanthin content (in mg/g) and fucoxanthin/n-3 LC-PUFA ratio (in w/w) of the different mixtures of *Phaeodactylum* total lipid extract and fish oil. Fucoxanthin was further highlighted because of its abundance and its importance in former research [8,263]. While other antioxidants may also play their role [260] fucoxanthin was thus considered as the major antioxidant. The reported values are measured values rather than calculated ones. This explains the apparently higher n-3 LC-PUFA value for the 'Mixture 1/99' than for 'Pure Fish oil' falling within the error margin.

Table 5.3: Characterization of the different *Phaeodactylum* total lipid extract – Fish oil mixtures in terms of n-3 LC-PUFA and fucoxanthin

System	N-3 LC-PUFA (mg/g)	Fucoxanthin (mg/g)	Fucoxanthin/n-3 LC-PUFA ratio (w/w)
Pure Phaeodactylum total lipid extract	117 (±1)	32.4 (±0.4)	0.277 (±0.003)
Mixture 79/21	145 (±3)	24.06 (±0.04)	0.1663 (±0.0003)
Mixture 49/51	165 (±4)	16.7 (±0.3)	0.101 (±0.002)
Mixture 6/94	222 (±3)	1.73 (±0.04)	0.0078 (±0.0002)
Mixture 1/99	232 (±3)	0.211 (±0.004)	0.00091 (±0.00002)
Pure Fish oil	227 (±6)	-	-

Since the fish oil has a higher n-3 LC-PUFA content than the *Phaeodactylum* total lipid extract (respectively 227 and 117 mg/g) it should not surprise that the n-3 LC-PUFA content increases as relatively more fish oil was used. The fucoxanthin content on the other hand decreases by blending more fish oil due to its absence of carotenoids. Therefore also the fucoxanthin/n-3 LC-PUFA ratio decreases from which it is clear that mixing the *Phaeodactylum* total lipid extract and fish oil in the amounts that were described in Table 5.1 led to different orders of magnitude of the fucoxanthin/n-3 LC-PUFA ratio. This formed an interesting starting point to investigate the oxidative stability of the different systems.

5.3.2.2. PRIMARY OXIDATION

Figure 5.4 shows the primary oxidation results of the six different systems. A clear separation in ΔPV progression between 'Pure *Phaeodactylum* total lipid extract', 'Mixture 79/21' and 'Mixture 49/51' on the one hand, and 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' on the other hand was observed. The first three showed a rather small, insignificant increase in ΔPV during storage, indicating that primary oxidation was only minor or absent. After 10 weeks, increases of respectively 52 (±33), 19 (±4) and 21 (±13) meq hydroperoxides/kg lipids were obtained. For the last three systems, much higher ΔPV values were detected, with a significant increase towards a maximum already after 4 days of storage, followed by a significant decrease after 8 days. Since a clear and early primary oxidation peak was observed, with ΔPV maxima of respectively 341 (±108), 402 (±79) and 491 (±46) meq hydroperoxides/kg lipids for 'Mixture 6/94', 'Mixture 1/99' and 'Pure fish oil', these systems were only analyzed during 4 weeks. Based on these results it could be stated that regarding primary oxidation 'Mixture

79/21' and 'Mixture 49/51' were in line with the pure *Phaeodactylum* extract of which the tendency not to lead to high ΔPV values was comparable with former research following primary oxidation during storage of *Phaeodactylum* hexane:isopropanol (3:2) extracts [81]. 'Mixture 6/94' and 'Mixture 1/99' on the other hand were in line with the pure fish oil of which a clear primary oxidation peak showed its high oxidative susceptibility. Comparing the primary oxidation results of the pure fish oil with those of Part A (3.1.1.), it was clear that the ΔPV peak occurred sooner. This could be attributed to the fact that in Part B amounts of 10 mg/vial instead of 1 g/vial were used, leading to a higher oxygen to bulk oil availability, explaining the accelerated oxidation processes [142]. Ryckebosch et al. [81] also observed a primary oxidation peak after 7 days (as the first time point) for refined fish oil being stored at 37 °C in amounts of 10 mg/vial with a weekly PV analysis.

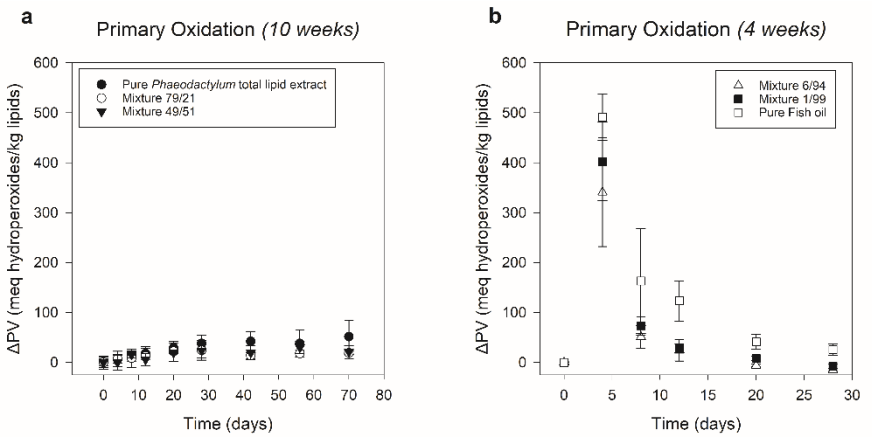


Figure 5.4: Primary oxidation of six different '*Phaeodactylum* total lipid extract – fish oil' systems during storage at 37 °C. 'Pure *Phaeodactylum* total lipid extract', 'Mixture 79/21' and 'Mixture 49/51' (respectively possessing 100.0%, 78.6% and 49.2% (w/w) of *Phaeodactylum* total lipid extract) were stored for 10 weeks (a). 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' (respectively possessing 6.4%, 0.7% and 0.0% (w/w) of *Phaeodactylum* total lipid extract) were stored for 4 weeks (b). The PV (mean ±sd, n=3) is expressed as delta values, representing the relative increase or decrease compared to day zero.

5.3.2.3. SECONDARY OXIDATION

The clear separation regarding the course of primary oxidation between 'Pure *Phaeodactylum* total lipid extract', 'Mixture 79/21' and 'Mixture 49/51' on the one hand, and 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' on the other hand was also visible for the secondary oxidation products, as can be seen in Figure 5.5.

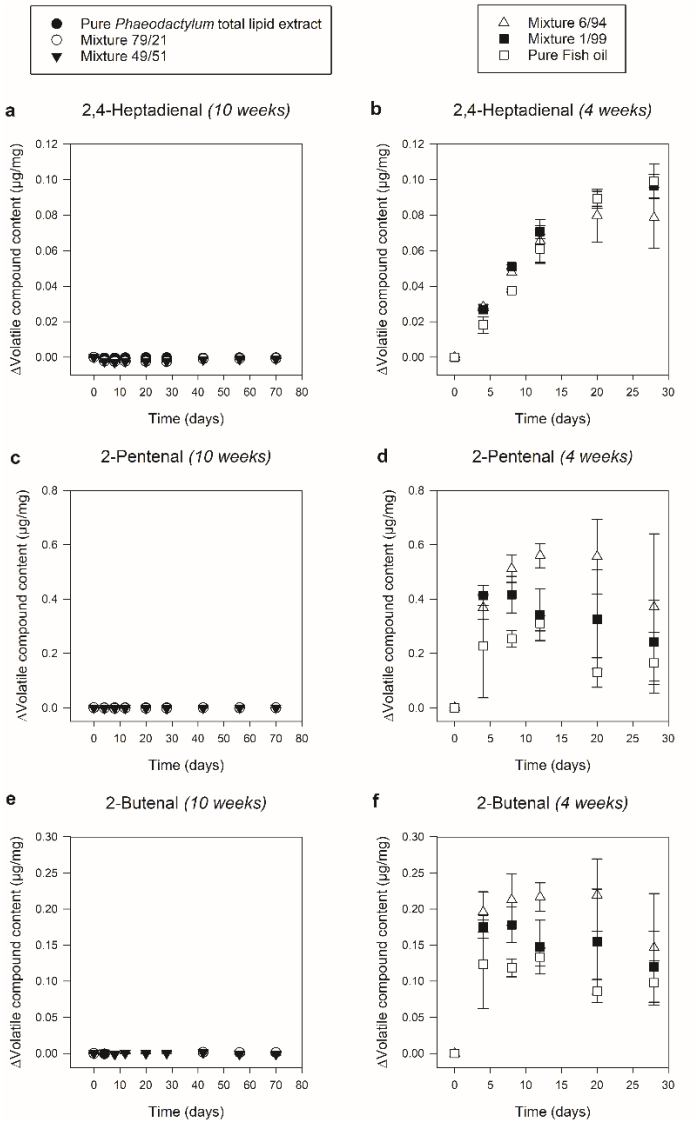


Figure 5.5: Secondary oxidation of six different '*Phaeodactylum* total lipid extract – fish oil' systems during storage at 37 °C. 'Pure Phaeodactylum total lipid extract', 'Mixture 79/21' and 'Mixture 49/51' (respectively possessing 100.0%, 78.6% and 49.2% (w/w) of *Phaeodactylum* total lipid extract) were stored for 10 weeks (left). 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' (respectively possessing 6.4%, 0.7% and 0.0% (w/w) of *Phaeodactylum* total lipid extract) were stored for 4 weeks (right). The content of volatile compounds originating from lipid oxidation [2,4-heptadienal (a and b), 2-pentenal (c and d), 2-butenal (e and f)] in µg/mg (mean ±sd, n=3) is expressed in delta values, representing the relative increase or decrease compared to day zero.

Figure 5.5 shows the progression of 2,4-heptadienal, 2-pentenal and 2-butenal as secondary n-3 LC-PUFA oxidation indicators. For all three compounds the 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' systems showed a clear, significant increase (of respectively ca. 0.02 µg/mg, ca. 0.3 µg/mg and ca. 0.15 µg/mg) already after 4 days of storage. Other than in Part A this significant increase of secondary oxidation products did not appear later than that of the primary oxidation products. Again, the relatively higher amount of oxygen as compared with the amount of bulk lipids could have led to accelerated oxidation, making the differentiation between the onset of primary and secondary oxidation less clear. The fact that also for the secondary oxidation products a clear, significant increase was already observed after the first time point, indicated thorough lipid oxidation for all three systems. The 'Pure *Phaeodactylum* total lipid extract', 'Mixture 79/21' and 'Mixture 49/51' systems on the other hand showed a flat course throughout 10 weeks of storage for all three compounds. This indicated the absence of clear secondary lipid oxidation, which is in line with the *Phaeodactylum* hexane:isopropanol (3:2) extract in the research of Ryckebosch et al. [81] and which also corresponds with the above described small increase in primary oxidation products for these three systems. However, other than for the primary oxidation results, Tukey analysis showed that for the 2,4-heptadienal and 2-pentenal content a small but significant decrease (of ca. 0.002 µg/mg) occurred after the first time point. Similarly as described for *Nannochloropsis* biomass by Gheysen et al. [8], a reason for this reduction could be that volatile organic compounds (VOCs), previously formed *in vivo* in *Phaeodactylum* and co-extracted in the total lipid extract, were already present at the start of the storage experiment and decomposed during storage. Although in literature 2,4-heptadienal and 2-pentenal are not specifically mentioned as known *Phaeodactylum* VOCs [264], Pohnert & Boland [265] described the *in vivo* formation of lipid oxidation derived unsaturated aldehydes (encompassing 2,4-heptadienal and 2-pentenal) as a defense mechanism for diatoms, which would explain their presence. Anyhow it is clear that this phenomenon (although significant) was only minor compared to the clear secondary lipid oxidation in the 'Mixture 6/94', 'Mixture 1/99' and 'Pure Fish oil' systems.

5.3.2.4. OXIDATIVE STABILITY AND ANTIOXIDATIVE CAPACITY

The results of both primary (5.3.2.2) and secondary oxidation (5.3.2.3) showed a clear separation in oxidative stability between the different systems. 'Mixture 79/21' and 'Mixture 49/51' (containing respectively 78.6% and 49.2% (w/w) of *Phaeodactylum* total lipid extract) have shown to be equally stable as the pure

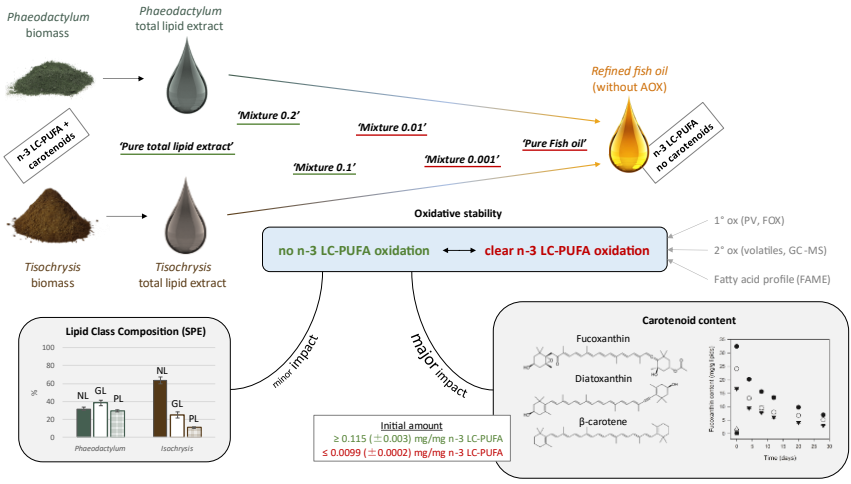
Phaeodactylum extract despite the presence of respectively 21.4% and 50.8% (w/w) of oxidatively susceptible fish oil. On the other hand 'Mixture 6/94' and 'Mixture 1/99' (containing respectively 93.6% and 99.3% (w/w) of fish oil) were shown to be equally unstable as the pure fish oil despite the presence of respectively 6.4% and 0.7% (w/w) of *Phaeodactylum* total lipid extract. This again clearly showed that apart from the presence of lipophilic antioxidants from the *Phaeodactylum* total lipid extract, of which the role in keeping *Phaeodactylum* lipids stable was already suggested in former research [8,81,260,263] and could again be observed in the oxidative stability of the (n-3 LC-PUFA rich) *Phaeodactylum* total lipid extract of the current research, also their amount plays a role in determining lipid oxidation to be either absent or far advanced.

Revisiting the fucoxanthin/n-3 LC-PUFA ratio of the different systems (5.3.2.1.), it could be stated that systems with a fucoxanthin/n-3 LC-PUFA ratio ≥ 0.101 showed to be oxidatively stable, whereas systems with a ratio ≤ 0.0078 led to strong lipid oxidation. In this regard the fact that 2.5% *Phaeodactylum* biomass addition to the fish oil did not show inhibition of oxidation (Part A) is not surprising, since this gave rise to a fucoxanthin/n-3 LC-PUFA ratio of only 0.00007 (±0.00002) in the oil phase. These findings therefore confirmed the hypothesis of antioxidant concentrations being too low as compared to n-3 LC-PUFA (as the oxidizable substrate) to have an antioxidative effect although being present in similar absolute concentrations as in former research where an antioxidative effect had been described [257]. Interesting to remark is that regarding the fucoxanthin migration to the oil phase in Part A, even a full transfer would not have led to a fucoxanthin/n-3 LC-PUFA ratio associated with oxidative stability. Therefore, no effort should be made in trying to improve antioxidant migration by additional treatments in trying to obtain an antioxidative effect of 2.5% *Phaeodactylum* biomass addition to fish oil. Moreover, significantly increasing the biomass concentration would also not lead to the fucoxanthin/n-3 LC-PUFA ratios of interest. An extreme, tenfold increase to 25% *Phaeodactylum* biomass for example would, whether or not feasible, give rise to a fucoxanthin/n-3 LC-PUFA ratio of 0.0007 (±0.0002) maximally (assuming a similar migration). Unlike other former research adding microalgal biomass as an antioxidant to bulk oil, of which the antioxidative effects of Morsy et al. [254] (*Arthrospira* addition to olive oil) were the most convincing, it is clear that *Phaeodactylum* biomass addition to stabilize fish oil thus has no potential.

5.4. CONCLUSIONS

In conclusion it can be stated that *Phaeodactylum* biomass (2.5 % (w/w)) was not capable of inhibiting n-3 LC-PUFA oxidation in fish oil, since the progression of both primary and secondary oxidation products showed strong lipid oxidation with and without biomass addition. *Phaeodactylum* carotenoids were however shown to be present in the fish oil phase after mixing, but these did not suffice to induce oxidative stability, since apart from their presence also their relative amounts as compared to the n-3 LC-PUFA content were of importance. This was clearly shown in the second experimental set-up in which the oxidative stability of *Phaeodactylum* total lipid extract and fish oil was followed after being mixed in different proportions leading to different carotenoid to n-3 LC-PUFA ratios. Systems with a fucoxanthin/n-3 LC-PUFA ratio ≥ 0.101 were associated with oxidative stability, whereas the systems with a ratio ≤ 0.0078 showed extreme lipid oxidation. Apart from explaining the absence of oxidation inhibition in case of 2.5% (w/w) *Phaeodactylum* biomass addition, this also showed that tenfold bigger amounts of biomass (whether practically feasible or not) would not be capable of obtaining this.

CHAPTER 6 - ANTIOXIDATIVE CAPACITY OF MICROALGAL CAROTENOIDS FOR STABILIZING N-3 LC-PUFA RICH OIL



Adapted from: Demets, R., Gheysen, L., Van Loey, A., & Foubert, I. (2023). Antioxidative capacity of microalgal carotenoids for stabilizing n-3 LC-PUFA rich oil: Initial quantity is key. *Food Chemistry*, 406 (135044).

6.1. INTRODUCTION

Still aiming to get further insight into the antioxidative capacity of microalgal carotenoids (more specifically those of *Phaeodactylum*) for stabilizing n-3 LC-PUFA, the current chapter built on the insights of Chapter 5 in two different ways: by considering the impact of lipid class composition and by (again) investigating the carotenoid composition throughout storage.

Regarding the impact of lipid class composition on lipid oxidation, it was reported by Lyberg et al. [266] and Yamaguchi et al. [267] that n-3 LC-PUFA are more oxidatively stable when incorporated in respectively PLs or GLs rather than in neutral lipids (NLs). PLs are also known to possess antioxidative properties themselves [144]. The fact that *Phaeodactylum* oil contains ca. one third of PLs, next to ca. one third of GLs and ca. one third of NLs [92], could therefore explain part of the stabilization effect that was observed in Chapter 5 when mixing *Phaeodactylum* total lipid extract with refined fish oil, only consisting of NLs. Elaborating on the impact of lipid class composition towards n-3 LC-PUFA oxidation could therefore give further insight into the effective role and antioxidative capacity of microalgal carotenoids: are carotenoids key for obtaining oxidative stability or does the presence of polar lipids also play an essential role?

The second aspect leading to more insight into the microalgal carotenoids' potential for n-3 LC-PUFA oxidation inhibition is analyzing them throughout storage rather than only considering their initial content, an approach that was also followed for the aqueous systems in Chapters 3 and 4. Carotenoids oxidatively degrade throughout storage which partly explains their antioxidative action [209]. Considering their presence throughout storage can therefore be clarifying for their role in causing oxidative stability. Moreover, different degradation kinetics for different carotenoids could give insight into the importance of carotenoid type. Since former research gave different outcomes on the antioxidant capacity of different types of carotenoids depending on the evaluation method used [190] it is difficult to state whether they are equally capable for oxidation inhibition or not.

In addressing both aspects, the experimental set-up of the second part of Chapter 5 (Part B) was expanded. Firstly, for studying the effect of lipid class composition, *Tisochrysis lutea* was selected (next to *Phaeodactylum* sp.) as another photoautotrophic microalga to get a total lipid extract from, rich in both n-3 LC-PUFA and fucoxanthin (as major carotenoid). *Tisochrysis* is however known to contain a bigger fraction of NLs (ca. 60%) compared to *Phaeodactylum* (ca.

30%) [92,268] enabling investigation of the lipid class impact. Apart from considering pure *Tisochrysis* total lipid extract and pure fish oil, they were mixed in such proportions that similar fucoxanthin/n-3 LC-PUFA ratios were reached as for the *Phaeodactylum* total lipid extract – fish oil mixtures (0.2, 0.1, 0.01 and 0.001). It was hypothesized that mixtures with similar fucoxanthin/n-3 LC-PUFA ratios would give rise to less oxidatively stable oils for *Tisochrysis* due to their lower amount of polar lipids. Secondly, to get further insight into the carotenoids' role, their content was again analyzed throughout storage for all samples (on top of primary and secondary lipid oxidation products). The fact that *Tisochrysis* contains similar carotenoids but in slightly different proportions (diatoxanthin and β-carotene being relatively more abundant as compared to fucoxanthin as major carotenoid [8]) gave another point of interest for investigating their relative importance when comparing the oxidative stability of systems with similar fucoxanthin/n-3 LC-PUFA ratios. Overall the research aimed at elucidating whether the fucoxanthin/n-3 LC-PUFA ratio is able to explain oxidative stability in n-3 LC-PUFA rich oils (as postulated in Chapter 5) or whether other aspects like lipid class composition play an important role. Finally, it should be stressed that obtaining further fundamental insight into the carotenoids' antioxidative role was the main objective rather than (already) developing a food grade application with antioxidative microalgal carotenoids. The latter could however be a target for further research taking the insights of the current one into account.

6.2. MATERIALS AND METHODS

All solvents used for preparing and analyzing the samples were HPLC- or GC-grade. Acetonitrile, chloroform, hexane and methanol were purchased from Carl Roth (Karlsruhe, Germany), ethyl acetate and toluene from VWR (Fontenay-sous-Bois, France).

6.2.1. RAW MATERIALS

Two different microalgae were used: *Phaeodactylum* and *Tisochrysis*, purchased as freeze-dried biomass from respectively Archimede Ricerche (Camporosso, Italy) (*Phaeodactylum* sp.) and Proviron (Hemiksem Belgium) (*Tisochrysis lutea*). They respectively contained 20.0 (±0.3) and 15.4 (±0.2) mg n-3 LC-PUFA, 5.3 (±0.5) and 5.5 (±0.2) mg fucoxanthin, 0.6 (±0.1) and 1.07 (±0.03) mg diatoxanthin and 0.48 (±0.02) and 0.55 (±0.01) mg β-carotene per g dry biomass (determined upon receipt according to the analyses as described below). INVE (Baasrode, Belgium) kindly provided a fresh, refined fish oil rich in n-3 LC-PUFA (243 (±1) mg/g) without added antioxidants nor carotenoids. PV (as determined by INVE) was 4.13 meq hydroperoxides/kg oil. N-3 LC-PUFA and carotenoid content were also determined using the analyses as described below. All raw materials were stored at -80 °C until use.

6.2.2. EXPERIMENTAL SET-UP

The experimental set-up consisted of two similar parts, one for each microalga, in which the lipids from the microalgal biomass were extracted and subsequently mixed with fish oil in different proportions to obtain six different systems (including both pure total lipid extract and pure fish oil). All twelve systems (in total) were characterized and exposed to a storage experiment. Figure 6.1 gives a graphical representation.

6.2.2.1. TOTAL LIPID EXTRACTION

For both *Phaeodactylum* and *Tisochrysis* a total lipid extract was obtained by performing a big scale chloroform:methanol (1:1) extraction on the microalgal biomass based on the procedure of Ryckebosch et al. [108]. The exact proceedings were described in 5.2.2.2. (part i). Chloroform:methanol (1:1) rather than a food grade solvent was used, since the main objective was to get more insight into the antioxidative capacity of lipophilic microalgal antioxidants before aiming at a practical application. For getting a full extraction of those, chloroform:methanol (1:1) has proven to be the best solvent [108].

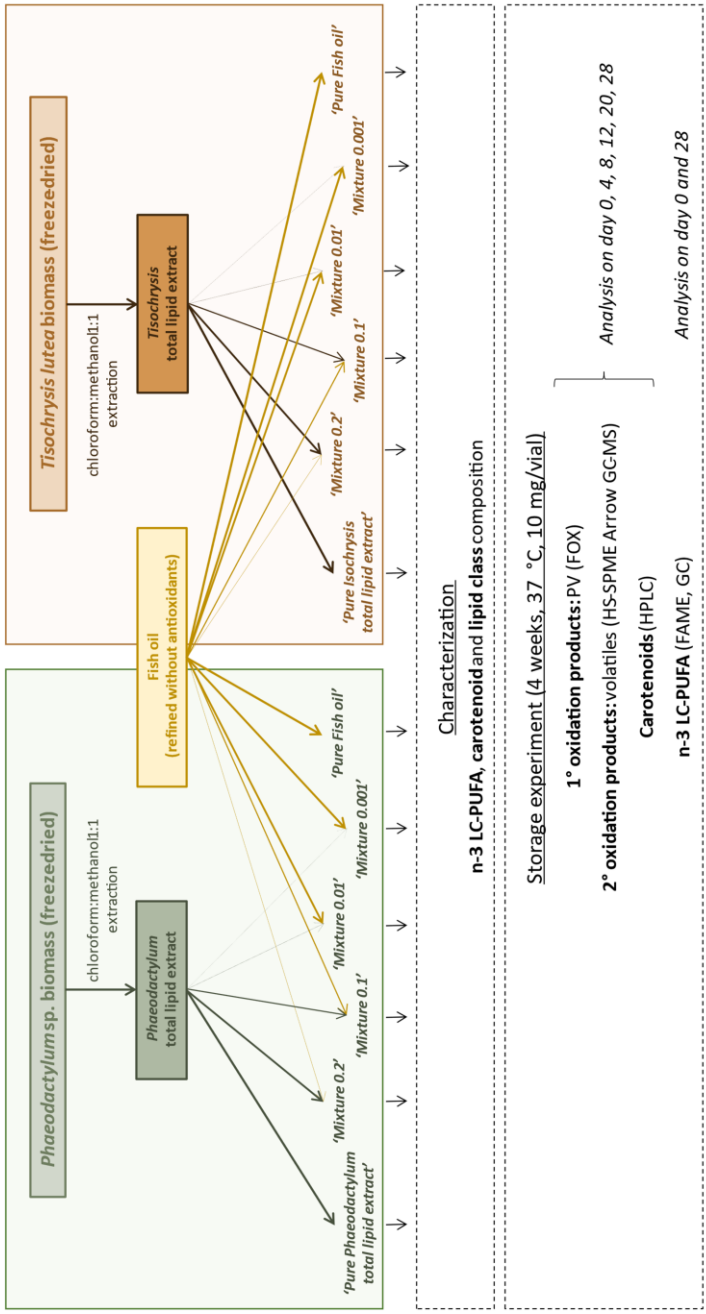


Figure 6.1: Experimental set-up

6.2.2.2. FORMULATION AND CHARACTERIZATION

For both algae, six different systems were formulated starting from the total lipid extract and the fish oil. For *Phaeodactylum* this concerned two systems of the pure oils (identified as 'Pure *Phaeodactylum* total lipid extract' and 'Pure Fish oil') and four mixtures containing respectively 78.6, 49.2, 6.4 and 0.7 w/w% of *Phaeodactylum* total lipid extract (identified as the *Phaeodactylum* 'Mixture 0.2', 'Mixture 0.1', 'Mixture 0.01' and Mixture 0.001' systems). For *Tisochrysis* also the pure oils were considered (identified as 'Pure *Tisochrysis* total lipid extract' and 'Pure Fish oil') and the remaining four (mixed) systems consisted of respectively 78.9, 56.4, 9.2 and 1.0 w/w% of the *Tisochrysis* total lipid extract (identified as the *Tisochrysis* 'Mixture 0.2', 'Mixture 0.1', 'Mixture 0.01' and 'Mixture 0.001' systems). These proportions were slightly different from the *Phaeodactylum* total lipid extract – fish oil mixtures because a similar fucoxanthin/n-3 LC-PUFA ratio was aimed for. This ratio is also being represented in the nomenclature of the mixtures. To ensure homogeneity when preparing the different mixed systems, the pure oils were dissolved in chloroform:methanol (1:1) before mixing. All systems were characterized in triplicate in terms of n-3 LC-PUFA content, carotenoids and lipid class composition according to the analyses as described below. To do so, part of the system was taken aside and dry-flushed with nitrogen. The obtained samples were stored at -80 °C until analysis

6.2.2.3. STORAGE EXPERIMENT

A storage experiment of 4 weeks at 37 °C was set up for all systems to assess both oxidative stability (by analyzing primary and secondary oxidation products) and carotenoid degradation (by analyzing carotenoid composition) throughout storage. Analyses were performed in triplicate for six different time points (day 0, 4, 8, 12, 20 and 28). Furthermore, the fatty acid profile of the different systems was determined at the start and end point (day 0 and day 28) of storage (also in triplicate). Storage was carried out in quantities of 10 mg in 20 mL screw-capped amber vials leading to independent samples for each of the different analyses. To obtain these quantities in the different vials, the systems were dissolved in chloroform:methanol (1:1) and distributed in solution followed by dry-flushing with nitrogen. The exact sample weights were determined gravimetrically using the weight difference of the vials with and without (dry) sample. After the different storage times of the storage experiment, the vials were kept at -80 °C until analysis

6.2.3. ANALYSES

6.2.3.1. FATTY ACID PROFILE

The fatty acid profile including the n-3 LC-PUFA content was determined by GC-FID after an acid-catalyzed methyl transesterification using the method as described in 3.2.2.6. C12:0 (Nu-Check Prep, Elysian, USA) was again used as internal standard.

6.2.3.2. CAROTENOIDS

Carotenoids were analyzed with HPLC-PDA according to the method as described in 3.2.2.7. with the sample preparation as described in 5.2.3.2.

6.2.3.3. LIPID CLASS COMPOSITION

Lipid class composition was determined in terms NL, GL and PL content (in weight%) using silica solid phase extraction (SPE) by the procedure as described in Balduyck et al. [269]. In short, a 20 mg lipid sample was dissolved in chloroform and brought onto an SPE column (polypropylene CHROMABOND® SiOH, Carl Roth, Karlsruhe, Germany) that was pre-rinsed with chloroform. The successive addition of 10 mL chloroform, 10 mL acetone and 10 mL methanol eluted respectively the NLs, GLs and PLs, of which the amount could be gravimetrically determined after solvent evaporation.

6.2.3.4. PRIMARY OXIDATION PRODUCTS

The amount of primary oxidation products or PV, was again analyzed with the adapted FOX method as described in 3.2.2.4. To focus on the oxidation trend, the results were expressed as delta values, giving the relative change from the starting value on day zero, and this for all of the different systems.

6.2.3.5. SECONDARY OXIDATION PRODUCTS

Secondary oxidation was determined by analysis of volatiles with HS-SPME Arrow GC-MS according to the method as described in 3.2.2.5. Just like in Chapter 5, 2,4-heptadienal, 2-pentenal and 2-butenal were focused upon as markers for secondary oxidation of n-3 LC-PUFA. Standards of these compounds were obtained from Sigma-Aldrich (Steinheim, Germany) and Carl Roth (Karlsruhe, Germany) (for 2-butenal), and were used for both identification confirmation and quantification by setting up external calibration plots (spiking the standards in the matrix of interest). Hexanal d-12 (CDN Isotopes, J.H. Ritmeester B.V., Nieuwegein,

Netherlands) was spiked as an internal standard for each analysis. The oxidation trend was again considered by expressing the results as delta values, giving the relative change from the starting value on day zero, for each of the systems.

6.2.4. MODELING (CAROTENOID DEGRADATION)

To enhance comparison of the different carotenoids throughout storage, kinetic modeling of their degradation was applied. For this a first-order degradation model was shown to adequately model the carotenoid degradation data:

$$\frac{d[C]}{dt} = -k[C]$$

with [*C*] being the carotenoid concentration under consideration at storage time *t* (in days) and *k* the reaction rate constant (per day). Assuming *k* to be constant in time, the equation becomes as follows with integration to time:

$$[C] = [C]_0 \exp(-kt)$$

For all mixtures with a gradual decrease in carotenoid content, the kinetic parameter *k* was estimated for each carotenoid (fucoxanthin, diatoxanthin and β-carotene) by non-linear regression with SigmaPlot 14.0 Software (Systat Software Inc., California, USA). Modeling was performed on the absolute carotenoid content (in mg/g lipids).

6.2.5. STATISTICAL ANALYSIS

Several analyses were used for statistically evaluating the results, all of them with a significance level of 0.05 and with the use of JMP Pro 15.1.0 software (SAS Institute Inc., Cary, USA). For characterization, one-way ANOVA with a post hoc Tukey test was used to investigate significant differences between the different systems regarding n-3 LC-PUFA content, fucoxanthin/n-3 LC-PUFA ratio and lipid class composition in terms of NL, GL and PL. Differences between the corresponding *Phaeodactylum* and *Tisochrysis* systems were focused upon. For oxidation, also one-way ANOVA was used to evaluate the impact of storage time on both primary and secondary oxidation products with a post hoc Tukey test to investigate at which time points differences occurred. Furthermore, to investigate differences in n-3 LC-PUFA and palmitic acid content between the start and the end of storage for the different systems, and to investigate differences regarding diatoxanthin and β-carotene over fucoxanthin content for the 'Pure total lipid extract' and 'Mixture 0.01' systems, t-tests were used. Finally, to evaluate significant differences between the estimated carotenoid degradation rate constants, 95% confidence regions (mean ±2sd) were considered.

6.3. RESULTS AND DISCUSSION

6.3.1. CHARACTERIZATION

Table 6.1 gives an overview of the (initial) composition of the different systems. Apart from indicating the weight fractions of total lipid extract and fish oil used for formulating the different systems, it reports the measured n-3 LC-PUFA content (in mg/g), fucoxanthin/n-3 LC-PUFA ratio (in mg/mg) and lipid class composition (weight percentage of NLs, GLs and PLs).

The n-3 LC-PUFA content of both microalgal total lipid extracts (56.3 mg/g for *Tisochrysis* and 117 mg/g for *Phaeodactylum*) was lower than that of fish oil (ca. 235 mg/g), causing n-3 LC-PUFA content to increase when blending in relatively more fish oil throughout the different mixtures. The fact that the *Tisochrysis* total lipid extract contained a lower amount of n-3 LC-PUFA than the *Phaeodactylum* extract corresponds with former research reporting total lipid and n-3 LC-PUFA contents of both algae [270]. The *Tisochrysis* total lipid extract however contained a significantly higher amount of fucoxanthin relative to the amount of n-3 LC-PUFA (0.47 vs. 0.277). The mixed systems were aimed at having similar fucoxanthin/n-3 LC-PUFA ratios when comparing *Tisochrysis* with *Phaeodactylum* (with the ratio that was aimed for indicated in the naming). The 'Mixture 0.2' systems of both algae however differed from this premised value leading to significantly different ratios (respectively 0.1663 and 0.26), while the ratios of the other mixtures did not. Although diatoxanthin and β-carotene were also present for all systems apart from the pure fish oil, these carotenoids will be discussed further (in 6.3.2.4 and 6.3.3).

Regarding the lipid class composition, a clear difference could be observed between the *Tisochrysis* total lipid extract and the *Phaeodactylum* total lipid extract. Whereas *Phaeodactylum* roughly contained similar amounts of all three classes, NLs were the predominant class for *Tisochrysis* (64%) with PLs only being present in relatively small amounts (11.2%). This difference is also present for the 'Mixture 0.2' and the 'Mixture 0.1' systems where the mixtures with *Tisochrysis* total lipid extract led to significantly lower amounts of PLs and significantly higher amounts of NLs compared to the ones with *Phaeodactylum* total lipid extract. In the 'Mixture 0.01' and 'Mixture 0.001' systems this difference was not present anymore due to the high amount of fish oil determining the biggest share of the lipid class composition. The fish oil itself exclusively contained NLs as was expected since refined fish oil was being used [57], causing the amount of NLs and PLs to relatively increase and decrease when more fish oil was incorporated.

Table 6.1: Composition (% extract, % fish oil) and characterization in terms of n-3 LC-PUFA content, fucoxanthin/n-3 LC-PUFA ratio and lipid class composition of the different microalgal total lipid extract – fish oil systems. For n-3 LC-PUFA content, fucoxanthin/n-3 LC-PUFA ratio and lipid class composition, an asterisk (*) next to the values in the *Tisochrysis* systems indicate a significant difference with the corresponding *Phaeodactylum* systems.

System	Total lipid extract (w/w%)	Fish oil (w/w%)	n-3 LC-PUFA content (mg/g)	Fucoxanthin/n-3 LC-PUFA ratio (mg/mg)	Lipid class composition		
					NLs (%)	GLs (%)	PLs (%)
Pure *Phaeodactylum* total lipid extract	100.0	0.0	117 (±1)	0.277 (±0.003)	32 (±2)	38 (±3)	30 (±1)
Phaeodactylum 'Mixture 0.2'	78.6	21.4	145 (±3)	0.1663 (±0.0003)	47 (±6)	30 (±5)	22 (±8)
Phaeodactylum 'Mixture 0.1'	49.2	50.8	165 (±4)	0.101 (±0.002)	65 (±3)	19 (±1)	16 (±2)
Phaeodactylum 'Mixture 0.01'	6.4	93.6	222 (±3)	0.0078 (±0.0002)	91 (±3)	4 (±2)	5 (±1)
Phaeodactylum 'Mixture 0.001'	0.7	99.3	232 (±3)	0.00091 (±0.00002)	100 (±1)	0 (±1)	0 (±2)
Pure Fish oil (*Phaeodactylum* set-up)	0.0	100.0	227 (±6)	-	100	-	-
Pure *Tisochrysis* total lipid extract	100.0	0.0	56.3 (±0.5)*	0.47 (±0.02)*	64 (±4)*	25 (±3)*	11.2 (±0.8)*
Tisochrysis 'Mixture 0.2'	78.9	21.1	75 (±8)*	0.26 (±0.03)*	74 (±1)*	19 (±2)*	7.8 (±0.8)*
Tisochrysis 'Mixture 0.1'	56.4	43.6	132.8 (±0.5)*	0.108 (±0.006)	82 (±2)*	12 (±2)	6 (±1)*
Tisochrysis 'Mixture 0.01'	9.2	90.8	224 (±2)	0.0081 (±0.0001)	95 (±4)	3 (±5)	1.8 (±0.8)
Tisochrysis 'Mixture 0.001'	1.0	99.0	244 (±2)	0.00092 (±0.00004)	100 (±4)	0 (±3)	0 (±2)
Pure Fish oil (*Tisochrysis* set-up)	0.0	100.0	240 (±3)	-	100	-	-

6.3.2. LIPID OXIDATION

6.3.2.1. OXIDATIVE STABILITY (PRIMARY AND SECONDARY OXIDATION)

Figures 6.2 and 6.3 show the results of respectively the primary and the secondary oxidation products throughout storage for both the *Phaeodactylum* and the *Tisochrysis* systems. Just as reported in Chapter 5 for the '*Phaeodactylum* total lipid extract – fish oil mixtures', also the '*Tisochrysis* total lipid extract – fish oil mixtures' showed a clear separation regarding primary and secondary oxidation progression.

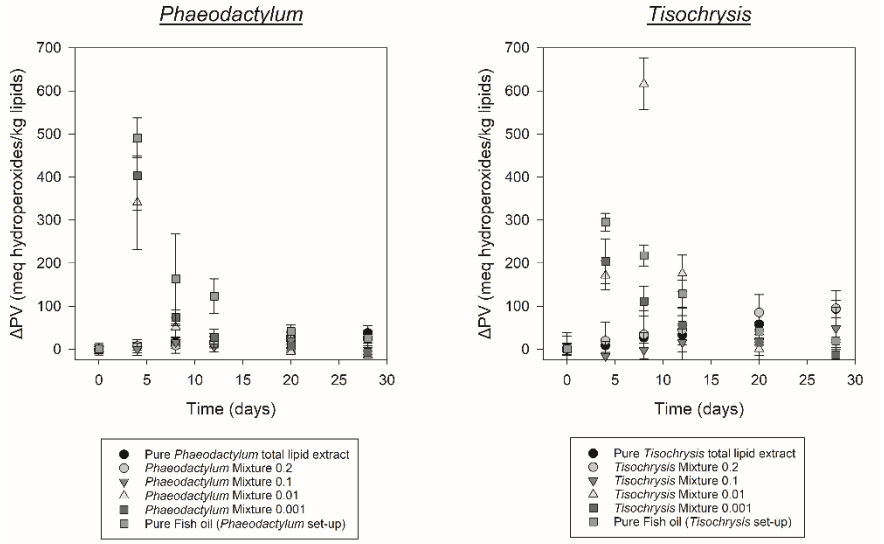


Figure 6.2: Primary oxidation of the different microalgal total lipid extract – fish oil systems during 28 days of storage at 37 °C. The *Phaeodactylum* systems (respectively containing 0.0%, 21.4%, 50.8%, 93.6%, 99.3% and 100.0% (w/w) of fish oil) are represented on the left, the *Tisochrysis* systems (respectively containing 0.0%, 21.1%, 43.6%, 90.8%, 99.0% and 100.0% (w/w) of fish oil) on the right. The PV (mean ±sd, n=3) is expressed as delta values (relative in- or decrease compared to day zero).

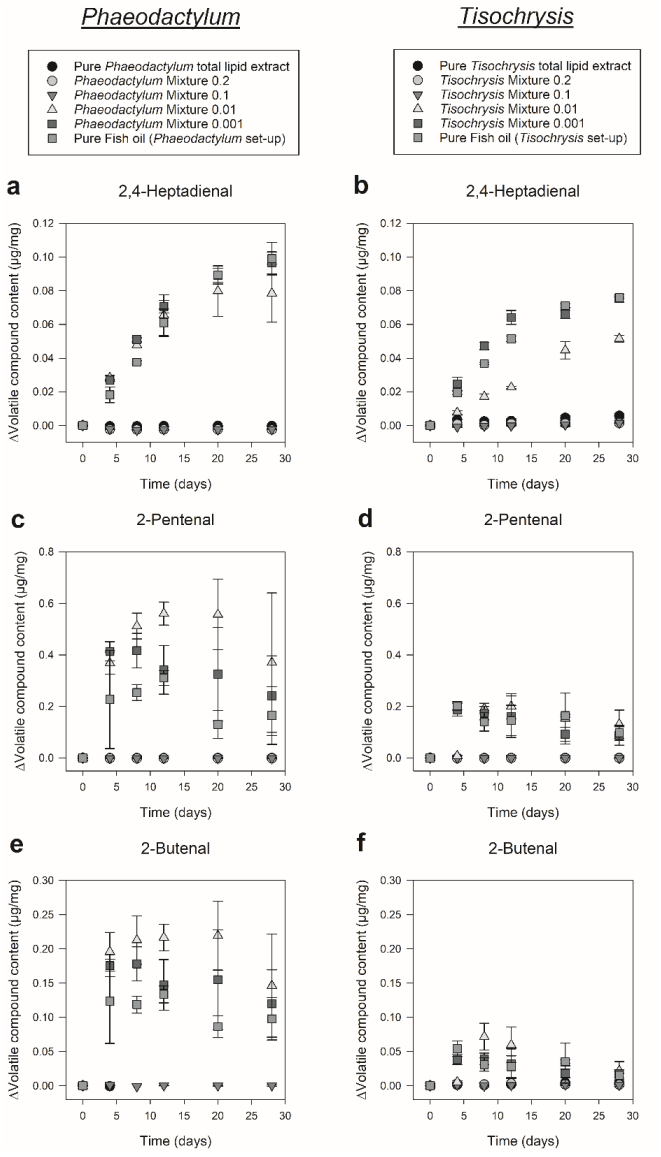


Figure 6.3: Secondary oxidation of the different microalgal total lipid extract – fish oil systems during 28 days of storage at 37 °C. The *Phaeodactylum* systems (respectively containing 0.0%, 21.4%, 50.8%, 93.6%, 99.3% and 100.0% (w/w) of fish oil) are represented on the left (subfigures a, c and e), the *Tisochrysis* systems (respectively containing 0.0%, 21.1%, 43.6%, 90.8%, 99.0% and 100.0% (w/w) of fish oil) on the right (subfigures b, d and f). 2,4-heptadienal, 2-pentenal and 2-butenal, marker volatile compounds from lipid oxidation, are respectively represented in subfigures 'a and b', 'c and d' and 'e and f'. Their content (mean ±sd, n=3) is expressed in delta values (relative in- or decrease compared to day zero).

The 'Mixture 0.2' and 'Mixture 0.1' systems (containing respectively 21.1% and 43.6% of fish oil for *Tisochrysis* and respectively 21.4% and 50.8% of fish oil for *Phaeodactylum*) showed an equally low oxidation progression as the pure microalgal total lipid extracts. Regarding primary oxidation, a slight increase throughout the whole storage experiment could be observed. Although the *Tisochrysis* systems tended to increase towards higher ΔPV values of 93 (±20), 95 (±41) and 48 (±48) meq hydroperoxides/kg lipids for respectively the 'Pure microalgal total lipid extract', 'Mixture 0.2' and 'Mixture 0.1' systems (as compared to 37 (±17), 24 (±20) and 24 (±15) for the corresponding *Phaeodactylum* systems), these increases were still not significant for the 'Mixture 0.2' and 'Mixture 0.1' systems and an order of magnitude lower than clearly oxidizing systems in former research analyzing primary oxidation with the same method [81,271]. Therefore, this indicated only minor primary oxidation. Also secondary oxidation was minor, demonstrated by the relatively flat courses of all three secondary n-3 LC-PUFA oxidation markers. Other than for the *Phaeodactylum* systems, a small, significant decrease of both 2,4-heptadienal and 2-pentenal after the first time point (addressed to the presence of these VOCs in the initial total lipid extract in Chapter 5) could not be observed for the *Tisochrysis* systems. On the contrary, small but significant increases could be noted for 2,4-heptadienal ('Pure total lipid extract' and 'Mixture 0.1' system) and 2-butenal ('Pure total lipid extract' and 'Mixture 0.2' system). However, considering the order of magnitude (ca. 0.006 µg/mg for 2,4-heptadienal and ca. 0.004 µg/mg for 2-butenal) these increases were only of minor relevance. The minor degrees of both primary and secondary oxidation were comparable with the oxidatively stable hexane:isopropanol (3:2) extracts of both *Phaeodactylum* and *Tisochrysis* in the research of Ryckebosch et al. [81] where also only small increases could be observed.

The 'Mixture 0.01' and 'Mixture 0.001' systems on the other hand (containing respectively 9.2% and 1.0% of microalgal total lipid extract for *Tisochrysis* and respectively 6.4% and 0.7% of microalgal total lipid extract for *Phaeodactylum*) showed thorough lipid oxidation comparable to the pure refined fish oil. For primary oxidation this was illustrated by a significant increase towards high ΔPV values (ranging from ca. 200 to ca. 600 meq hydroperoxides/kg lipids) followed by a significant decrease. Clear secondary oxidation was observed by a clear significant increase for all three volatile markers. However, although all three 'Mixture 0.01', 'Mixture 0.001' and 'Pure Fish oil' systems of the *Tisochrysis* set-up were clearly oxidatively unstable by showing these phenomena in terms of

primary and secondary oxidation products, the 'Mixture 0.01' system differed from the other two in showing a somewhat delayed oxidation. Whereas the maximal ΔPV value was on the first time point (4 days) for the 'Mixture 0.001' and 'Pure Fish oil' system, it was only observed at the second time point (8 days) for the 'Mixture 0.01' system. Also, 2-pentenal and 2-butenal only significantly increased after the second time point in the *Tisochrysis* 'Mixture 0.01' system while a clear, significant increase could already be observed after the first time point for the other oxidatively unstable systems. Also, the more gradual increase of 2,4-heptadienal was (although already being significant at the first time point) lagging behind in terms of order of magnitude. The fact that this difference could not be observed for the *Phaeodactylum* systems, with both 'Mixture 0.01' and 'Mixture 0.001' systems being equally unstable as the pure fish oil is interesting for further discussion linking oxidation with characterization (see sections 6.3.2.3 and 6.3.2.4). Anyhow, the progression of both primary and secondary oxidation products of the 'Mixture 0.01' system of both *Tisochrysis* and *Phaeodactylum* (showing delay or not), was still clearly different from the oxidative stability observed in the 'Mixture 0.1' and 'Mixture 0.2' systems

6.3.2.2. FATTY ACID PROFILE

Although the results of the primary and secondary oxidation progression were already conclusive regarding the oxidative stability of the different systems, the clear separation between minor and thorough lipid oxidation was confirmed by the results of the n-3 LC-PUFA and C16:0 content (as part of the fatty acid profile) at the beginning and end of storage. Figure 6.4 gives a graphical representation of the results of the n-3 LC-PUFA and C16:0 content. For the 'Mixture 0.2' and 'Mixture 0.1' systems of both *Phaeodactylum* and *Tisochrysis*, the n-3 LC-PUFA content after the full storage time (4 weeks) did not differ significantly from the initial value. Although the absence of a detectable n-3 LC-PUFA degradation is no guarantee for the absence of lipid oxidation since analyzing the fatty acid profile is considered as an insensitive method for lipid oxidation [223], the result coincided with the former findings of minor lipid oxidation for these systems. To the contrary, for the 'Mixture 0.01' and 'Mixture 0.001' systems of both algae, no n-3 LC-PUFA could be detected at the end of storage, indicating a full degradation of these fatty acids. Moreover, the C16:0 content, a fatty acid that is insensitive for lipid oxidation given the absence of unsaturation in the carbon chain, remained quite similar at the end point of storage for these system (with a decrease of maximally 7 (±1)%). Therefore the n-3 LC-PUFA degradation was a

clear confirmation of the far advanced stage of lipid oxidation of these systems, corresponding with the primary and secondary oxidation results above.

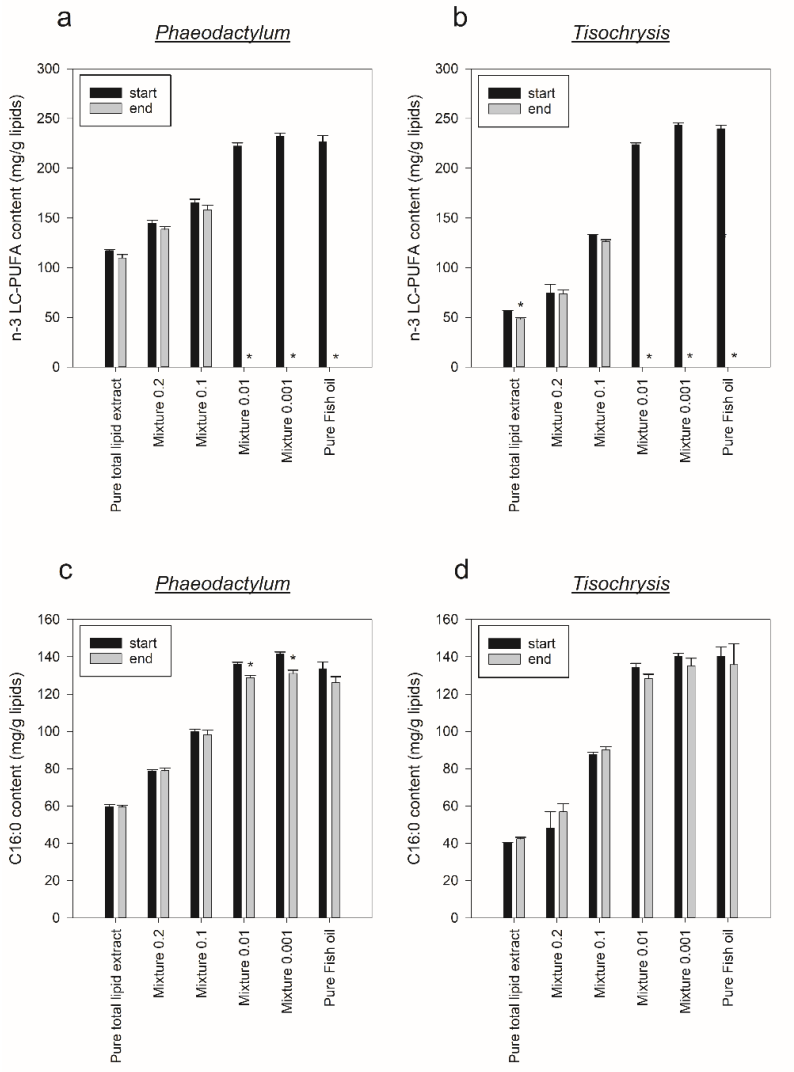


Figure 6.4: n-3 LC-PUFA and C16:0 content in mg/g lipids (mean ±sd, n=3) of the different microalgal total lipid extract – fish oil systems at the start and end of a 28 days storage period at 37 °C. The *Phaeodactylum* systems (respectively containing 0.0%, 21.4%, 50.8%, 93.6%, 99.3% and 100.0% (w/w) of fish oil) are represented on the left (n-3 LC-PUFA in subfigure a, C16:0 in subfigure c), the *Tisochrysis* systems (respectively containing 0.0%, 21.1%, 43.6%, 90.8%, 99.0% and 100.0% (w/w) of fish oil) on the right (n-3 LC-PUFA in subfigure b, C16:0 in subfigure d). Within each system, statistical difference between the start and end of storage are represented by an asterisk (*).

6.3.2.3. LINK LIPID CLASS COMPOSITION

From the above it is clear that formulating microalgal total lipid extract – fish oil mixtures with *Tisochrysis* instead of *Phaeodactylum* led to the same separation between either being oxidatively stable or not. Despite having significantly lower amounts of PLs (and higher amounts of NLs) (see section 6.3.1.) the *Tisochrysis* 'Pure total lipid extract', 'Mixture 0.2' and 'Mixture 0.1' systems remained as oxidatively stable as the corresponding systems with *Phaeodactylum* total lipid extract. Therefore the hypothesis of mixtures with similar fucoxanthin/n-3 LC-PUFA ratios but lower amounts of polar lipids leading to a lower oxidative stability has to be rejected. Although it cannot be stated that PLs are of no importance regarding the stability in the systems of interest (given their minor presence also in the *Tisochrysis* total lipid extract), the relative amount of fucoxanthin seemed to be more decisive. In the oxidatively unstable systems, the fucoxanthin/n-3 LC-PUFA is however also not completely determinant. The 'Mixture 0.01' system of *Tisochrysis* was characterized by a slower (yet finally thorough) oxidation than the 'Mixture 0.01' system of *Phaeodactylum* despite showing no significant difference in fucoxantin/n-3 LC-PUFA ratio (see section 6.3.1.). Nonetheless, the (insignificant) difference in lipid class composition between these systems was too low to be of explanatory value, and based on the antioxidative potential of PLs [144] and their relatively lower amounts in *Tisochrysis* total lipid extract the opposite finding would rather have been expected. Summarizing the above findings, it can be stated that the influence of lipid class composition was probably minor. It should be mentioned that the presence of any influence cannot be excluded given the fact that the corresponding systems of both microalgae not only differ in lipid class composition but also in other compounds originating from the microalgal extracts.

6.3.2.4. LINK INITIAL CAROTENOID COMPOSITION

An aspect that may be of explanatory value for the slower oxidation of the *Tisochrysis* 'Mixture 0.01' system (apart from the clear separation between the oxidatively stable 'Pure total lipid extract', 'Mixture 0.2' and 'Mixture 0.1' systems and the oxidatively unstable 'Mixture 0.01', 'Mixture 0.001' and 'Pure Fish oil' systems) is the abundance of other (minor) carotenoids. Next to fucoxanthin, diatoxanthin and β-carotene were also present in both the *Phaeodactylum* total lipid extract and the *Tisochrysis* total lipid extract. However, their content relative to the amount of fucoxanthin was significantly higher for *Tisochrysis* (0.177 (±0.005) mg diatoxanthin/mg fucoxanthin and 0.081 (±0.004) mg β-carotene/mg

fucoxanthin) than for *Phaeodactylum* (0.084 (±0.002) mg diatoxanthin/mg fucoxanthin and 0.050 (±0.005) mg β-carotene/mg fucoxanthin). The relative amount of both diatoxanthin and β-carotene to n-3 LC-PUFA for the 'Mixture 0.01' system of *Tisochrysis* was therefore also higher than for the 'Mixture 0.01' system of *Phaeodactylum* having an equal fucoxanthin/n-3 LC-PUFA ratio (see 3.1): respectively 0.00126 (±0.00003) mg diatoxanthin/mg n-3 LC-PUFA vs 0.00073 (±0.00003) mg diatoxanthin/mg n-3 LC-PUFA, and 0.00051 (±0.00001) mg β-carotene/mg n-3 LC-PUFA vs. no quantifiable amounts of β-carotene. As the antioxidative properties of these carotenoids have also been reported [190,272], their higher abundance may be a possible reason for the slower oxidation. To get a better insight into the role of the different carotenoids, their content throughout storage is discussed in the next section.

6.3.3. CAROTENOIDS THROUGHOUT STORAGE

6.3.3.1. DEGRADATION

Figure 6.5 shows the results of the carotenoid composition throughout storage for the different systems containing microalgal total lipid extract. The 'Pure Fish oil' systems are not shown since the refined fish oil did not contain any carotenoids. It is clear that all three carotenoids (fucoxanthin, diatoxanthin and β-carotene) showed a degradation during storage for all systems. For the 'Pure total lipid extract', 'Mixture 0.2' and 'Mixture 0.1' systems of both *Phaeodactylum* and *Tisochrysis*, the systems that were stable towards n-3 LC-PUFA oxidation (see 6.3.2.1.), this degradation proceeded gradually with a lower content being detected after every time point for every carotenoid. The oxidatively unstable 'Mixture 0.01' and 'Mixture 0.001' systems on the other hand showed an immediate degradation to non-quantifiable amounts on the first time point (4 days). Only for the *Tisochrysis* 'Mixture 0.01' system, a low amount of fucoxanthin (0.05 ±0.02 mg/g lipids) could still be detected after 4 days which however also fully degraded thereafter (8 days).

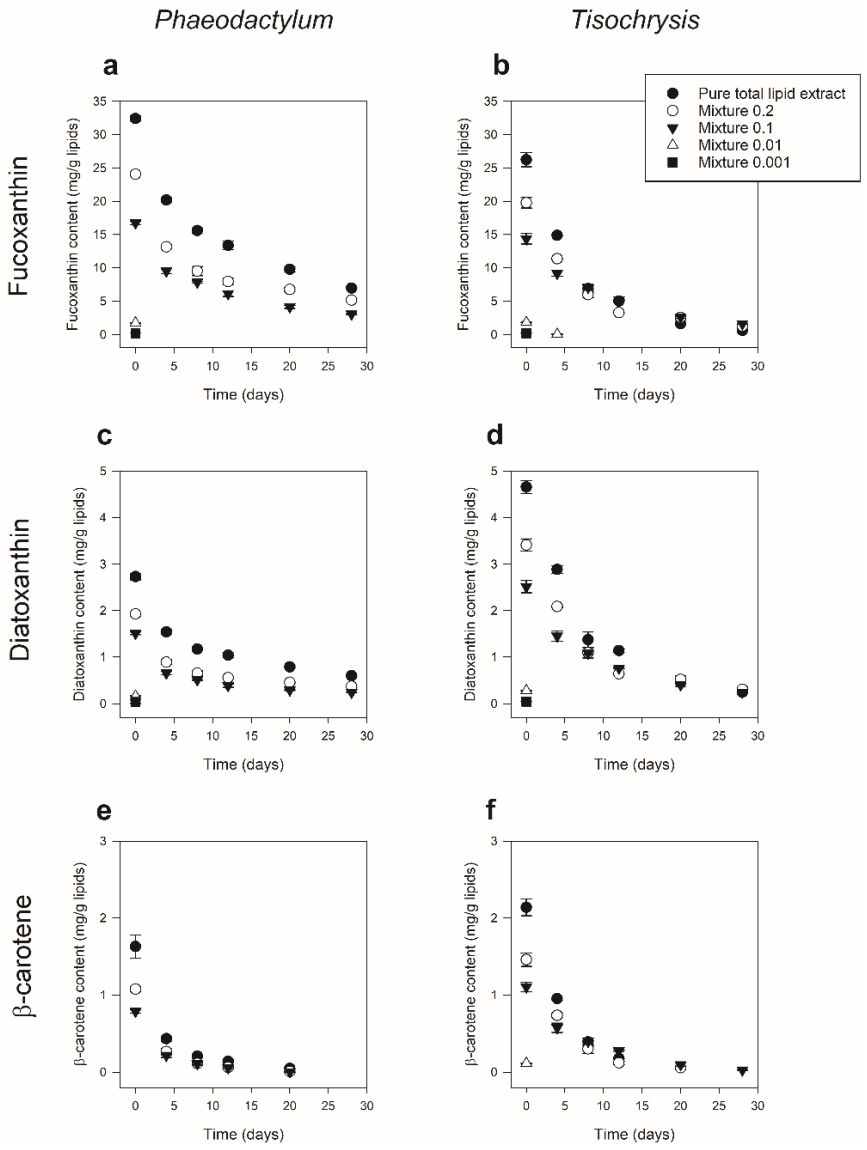


Figure 6.5: Evolution of fucoxanthin (subfigures a and b), diatoxanthin (subfigures c and d) and β-carotene (subfigures e and f) content in mg/g lipids (mean ±sd, n=3) during 28 days of storage at 37 °C for the different microalgal total lipid extract – fish oil systems. The 'Pure total lipid extract', 'Mixture 0.2', 'Mixture 0.1', 'Mixture 0.01' and 'Mixture 0.001' of *Phaeodactylum* (containing respectively 0.0%, 21.4%, 50.8%, 93.6% and 99.3% (w/w) of fish oil) are represented in subfigures a, c and e; those of *Tisochrysis* (containing respectively 0.0%, 21.1%, 43.6%, 90.8% and 99.0% (w/w) of fish oil) are represented in subfigures b, d and f. Carotenoids were absent in pure fish oil. Non-quantifiable amounts are not displayed.

The fact that, regarding carotenoid degradation, a similar separation between the different systems as for the oxidative stability could be made, showed the association between oxidative stability and carotenoid degradation. In the oxidatively stable systems carotenoids were present throughout the whole storage period, whereas they were absent in the oxidizing systems and this at the time point on which lipid oxidation could clearly be measured (on day 4 for the 'Mixture 0.01' and 'Mixture 0.001' systems of *Phaeodactylum* and the 'Mixture 0.001' system of *Tisochrysis*, and on day 8 for the more slowly oxidizing 'Mixture 0.01' system of *Tisochrysis*). Moreover, the degradation illustrates the carotenoids' antioxidative role. Given that heat, light and acids were absent during storage, a breakdown of carotenoids can either be explained by radical scavenging or by oxidative degradation [209]. The antioxidative effect of the first one is clear as it prevents lipid radicals from propagating into the autocatalytic chain process of lipid oxidation [186], but also the oxidative degradation can be seen as antioxidative since the oxygen in the near environment of both lipids and carotenoids is no longer available for reaction with the lipids upon reaction with the carotenoids. For the 'Pure total lipid extract', the 'Mixture 0.2' and the 'Mixture 0.1' systems, showing clear oxidative stability (no or minor increase in both primary and secondary oxidation products), the carotenoids were thus exerting an important antioxidative activity as shown by their degradation. Also, since this degradation was visible for fucoxanthin, diatoxanthin and β-carotene, the antioxidative role could be attributed to all three carotenoids. However it can be suggested that a sufficient content of β-carotene is not crucial for oxidative stability, since the systems in which β-carotene showed full degradation at the end of storage still showed no oxidation onset at all. With fucoxanthin and diatoxanthin still being present in those systems, this coincides with the findings of Liao & Hu [186] stating that xanthophylls are potent chain-breaking antioxidants in non-polar environments.

6.3.3.2. KINETICS (OXIDATIVELY STABLE SYSTEMS)

To further evaluate the differences between the different carotenoids and to investigate possible differences between the set-ups of the different algae, the degradation rate constants obtained after modeling the carotenoid degradation were compared. Since the systems showing clear lipid oxidation showed an immediate breakdown of carotenoids, this was only useful for the oxidatively stable systems. Figure 6.6 shows the different first-order degradation rate constants of those systems.

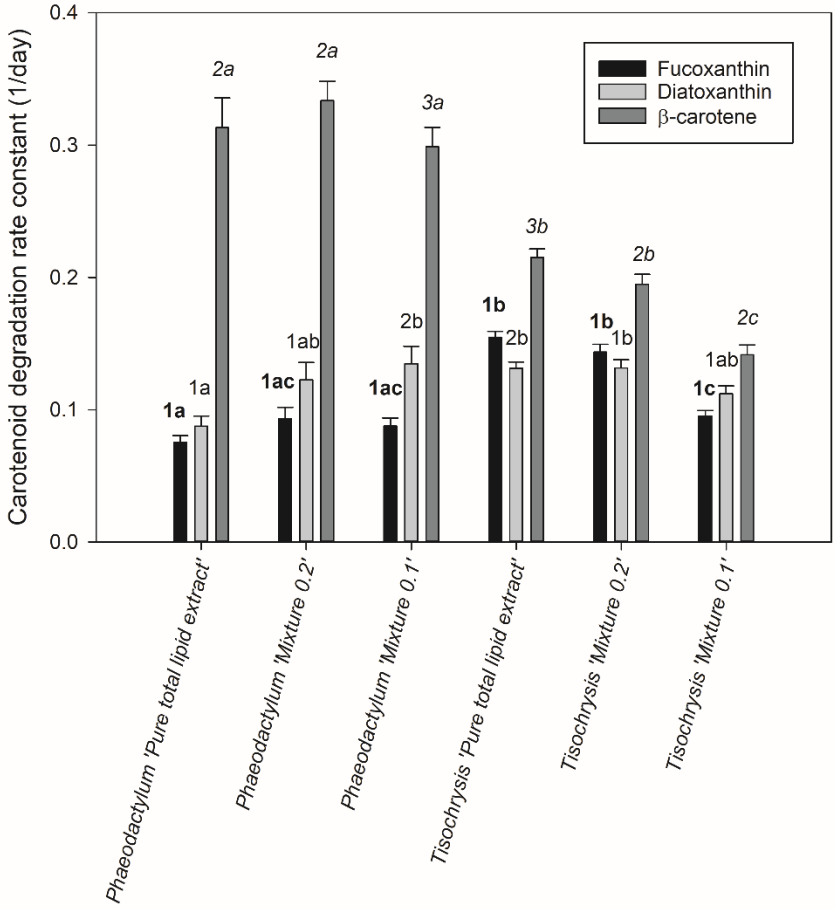


Figure 6.6: First-order degradation rate constants (in day^{-1}) of fucoxanthin, diatoxanthin and β-carotene at 37 °C in the 'Pure total lipid', 'Mixture 0.2' and 'Mixture 0.1' systems of *Phaeodactylum* and *Tisochrysis* (containing respectively 0.0%, 21.4%, 50.8% (w/w) of fish oil for *Phaeodactylum* and 0.0%, 21.1 and 43.6% (w/w) of fish oil for *Tisochrysis*). Significant differences between different carotenoids are indicated by a different number within each system. Significant differences between different systems are indicated by a different letter for each carotenoid.

For each system, the rate constant of β-carotene degradation was significantly higher than the one of both fucoxanthin and diatoxanthin. In other words, β-carotene was characterized by a faster breakdown. As mentioned above, this can be explained by both a higher radical scavenging activity or a faster oxidative degradation. It was reported by Martin et al. [236] that the extent to which

carotenoids intervene in the radical chain process depends on their chemical structure (end groups, chain length and number and position of methyl groups). Although fucoxanthin and diatoxanthin were not considered in that particular research, the high tendency of β-carotene to do so as compared to astaxanthin (a xanthophyll) was mentioned, which seems to coincide with the faster breakdown of β-carotene in the current research. A difference between fucoxanthin and diatoxanthin was less clear with non-significantly different degradation rate constants for most of the systems. Apart from the degradation kinetics being dependent on carotenoid type, also the microalgal extract seems to have an influence: for *Tisochrysis* the degradation rate constants were significantly lower for β-carotene and, apart from the 'Mixture 0.1' systems, significantly higher for fucoxanthin. These differences did however not have an influence on oxidative outcome (see section 6.3.2.1.).

Comparing the order of magnitude of the different degradation rate constants with former research is not straightforward since most research on modeling carotenoid degradation focused on thermal degradation [209,273] which is only relevant at temperatures that are clearly higher (60 – 240 °C) than the storage temperature (37 °C) in this research. Zhao et al. [274] did however model the degradation of fucoxanthin in canola oil at 37 °C, reporting a first-order degradation rate constant of 0.0014 (±0.0004) h^{-1}. Expressed per day this value equals 0.03 (±0.01) day^{-1}, which is clearly lower than the degradation rate constants observed in this study. This can probably be explained by the higher oxidation susceptibility of the n-3 LC-PUFA rich systems of the current research which could be associated with a higher tendency to form radicals [7] and therefore carotenoid breakdown, again confirming their antioxidative role.

6.3.3.3. OXIDATIVELY UNSTABLE SYSTEMS

Given the (almost) immediate degradation of carotenoids in the oxidatively unstable systems of which the initial carotenoid content was lower, it was interesting to investigate whether or not the above estimated degradation kinetics could explain the fast absence during storage. When calculating the expected carotenoid concentrations at the first time point (day 4) with the starting concentration of the (unstable) 'Mixture 0.001' systems, based on the kinetic models of the (stable) 'Mixture 0.01' systems, they were clearly higher than the observed carotenoid contents. Fucoxanthin for example was estimated to be present in amounts of ca. 1.25 mg/g lipids for both *Phaeodactylum* and *Tisochrysis* at the time point of 4 days, while in reality it respectively degraded

towards an amount that was non-quantifiable and an amount of 0.05 (±0.02) mg/g lipids. It can therefore be stated that in the systems showing clear lipid oxidation the absence of carotenoids at further time points is not only caused by a lower starting content, but also by a faster degradation as compared to the oxidatively stable systems. This faster degradation illustrates that the carotenoids are not capable of preventing oxidation in the same way as in the oxidatively stable systems, but rather co-oxidize within the propagating autoxidation process. This clearly shows the importance of having a sufficient initial amount of carotenoids relative to the amount of oxidizable substrate, in order for them to prevent n-3 LC-PUFA oxidation.

Taking into account that all three carotenoids exerted an antioxidative role in the oxidatively stable systems (as explained by their degradation in section 6.3.3.1.), the sum of carotenoids should be considered rather than the fucoxanthin content on its own (as suggested in Chapter 5). Based on the above results it could be stated that carotenoid contents of 0.115 (±0.003) mg/mg n-3 LC-PUFA or higher sufficed for giving rise to oxidatively stable systems. If on the other hand carotenoids are not abundant enough initially, they cannot avoid the autoxidation process and simply oxidize together with the lipids. This was the case for systems with a carotenoid content of 0.0099 (±0.0002) mg/mg n-3 LC-PUFA or lower. Although the 'Mixture 0.01' system of *Tisochrysis* was characterized by a somewhat delayed oxidation, which might be explained by a higher carotenoid abundance (0.0099 ±0.0002 mg/mg n-3 LC-PUFA) as compared to the directly oxidizing *Phaeodactylum* 'Mixture 0.01' system (0.0085 ±0.0002 mg/mg n-3 LC-PUFA) due to the higher abundance of diatoxanthin and β-carotene in systems with equal fucoxanthin amounts (see section 6.3.2.4.), this content did not suffice for preventing lipid oxidation.

6.4. CONCLUSIONS

In conclusion, it can be stated that microalgal carotenoids are capable of accomplishing oxidative stability in n-3 LC-PUFA rich oils, but that their initial quantity is decisive for the oil to be either oxidatively stable or unstable. The lipid class content on the other hand, although showing clear differences between *Tisochrysis* and *Phaeodactylum*, seemed to be of minor relevance disproving the hypothesis of *Tisochrysis* systems having a lower oxidative stability due to the lower amounts of polar lipids. A small delay in oxidation in the 'Mixture 0.01' system of *Tisochrysis* may be explained by the higher abundance of also the minor carotenoids diatoxanthin and β-carotene. The fact that all three carotenoids (fucoxanthin, diatoxanthin and β-carotene) were exerting an antioxidative effect was illustrated by their gradual degradation throughout storage for the oxidatively stable systems. β-carotene was characterized by a faster breakdown, possibly caused by a higher tendency to intervene within the radical chain process. The rate of carotenoid breakdown was however clearly faster for all carotenoids in the oxidatively unstable systems with an (almost) immediate degradation to non-quantifiable amounts. This illustrated that the carotenoids, when being present in too low amounts relative to the n-3 LC-PUFA (0.0099 ±0.0002 mg/mg n-3 LC-PUFA or lower), do not possess the same stabilizing capacities as compared to being present in high initial amounts (0.115 (±0.003) mg/mg n-3 LC-PUFA or higher).

CHAPTER 7 - GENERAL CONCLUSIONS

The current PhD research is situated within the research on microalgae as an alternative n-3 LC-PUFA source and has a strong focus on oxidative stability. As became clear in the literature review (Part I), several microalgae show potential to overcome both sustainability and stability issues in developing n-3 LC-PUFA rich food products: their cultivation offers an alternative for the overexploited global fish stock by providing a new n-3 LC-PUFA supply (Chapter 1), and their addition to food products (food enrichment) can lead to systems with only minor amounts of lipid oxidation (Chapter 2). With former research pointing out the importance of endogenous carotenoids in doing so [9], the general aim of the current research was to further unravel the endogenous carotenoids' antioxidative role and potential and, in this way, gain further knowledge on microalgal n-3 LC-PUFA stability. Throughout the different experimental research chapters (Part II) several insights could be gained. In what follows, these findings are summarized into multiple conclusions. A distinction is made between conclusions with a fundamental nature (7.1.) and conclusions towards practical applications (7.2.). Both sections end with a discussion on future research perspectives.

7.1. CONCLUSIONS WITH A FUNDAMENTAL NATURE

7.1.1. THE IMPORTANCE OF MICROALGAL CAROTENOIDS IN ENSURING N-3 LC-PUFA STABILITY

7.1.1.1. ANTIOXIDATIVE ROLE

First of all, throughout the storage experiments of the different experimental chapters, **the antioxidative action of microalgal carotenoids was confirmed**. As has been mentioned before, a degradation of carotenoids in the absence of heat and light can provide information about their antioxidative contribution. It indicates radical scavenging or oxidative degradation, both of which can be seen as antioxidative (respectively in a direct or indirect way) [209]. These conditions applied to Chapters 3 and 6, in which a degradation of **all (microalgal) carotenoids** was observed. Therefore, it could be stated that all carotenoids present had an antioxidative effect **in both biomass suspensions** (Chapter 3) **and bulk oil mixtures** (Chapter 6).

Moreover, the results of the carotenoid profile throughout storage showed a **clear link with the occurrence of lipid oxidation**, which only demonstrated their antioxidative role even more. Figure 7.1 gives a summarizing overview of this link between carotenoid degradation and lipid oxidation.

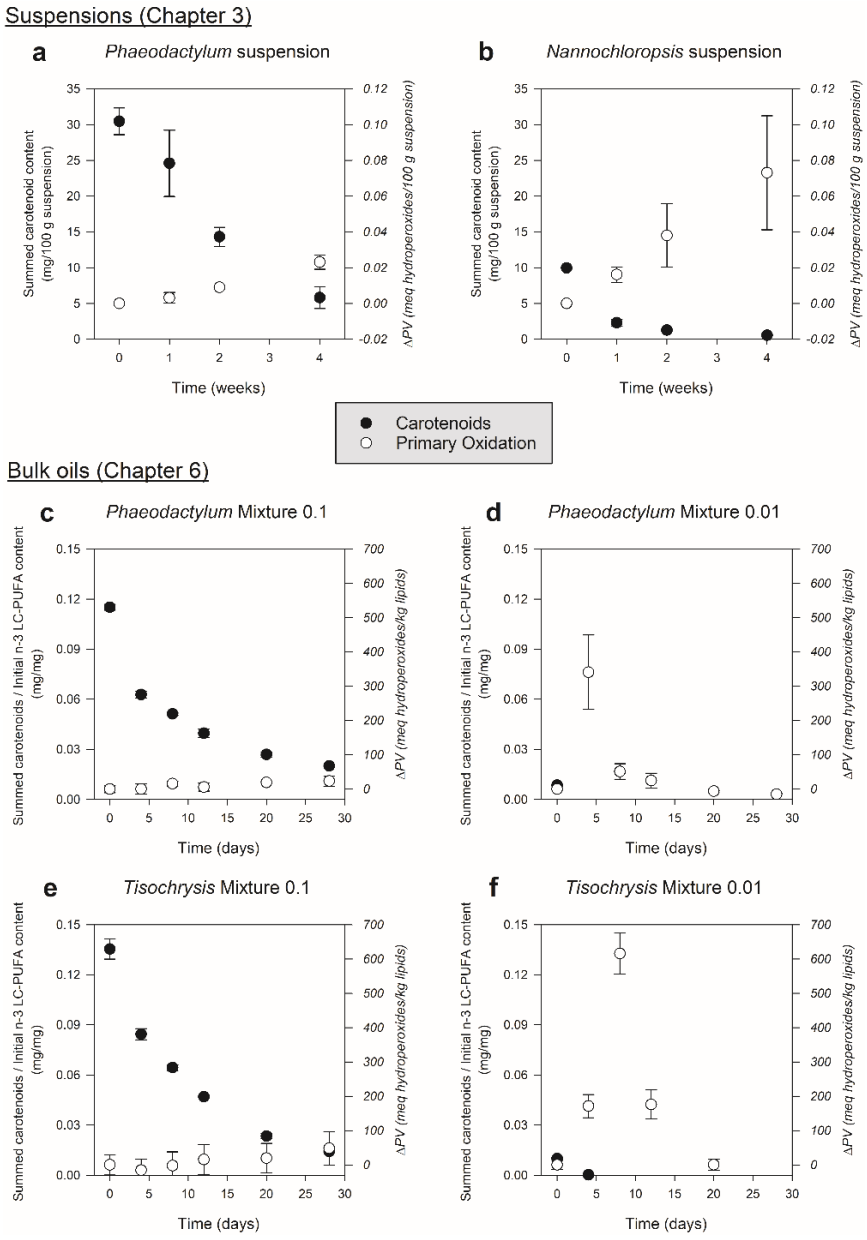


Figure 7.1: Carotenoid (black symbols) and primary oxidation (white symbols) evolution throughout storage for the *Phaeodactylum* (a) and *Nannochloropsis* (b) suspensions from Chapter 3, and the 'Mixture 0.1' and 'Mixture 0.01' systems of *Phaeodactylum* (c and d) and *Tisochrysis* (e and f) from Chapter 6. Non-quantifiable amounts (carotenoids) are not displayed.

For both suspensions of Chapter 3 (*Phaeodactylum* and *Nannochloropsis*) and the borderline (in terms of oxidative stability) bulk oil systems of Chapter 6 ('Mixture 0.1' [stable] and 'Mixture 0.01' [unstable] systems of both *Phaeodactylum* and *Tisochrysis*), the evolution of the summed carotenoid content and the primary oxidation products throughout storage were simultaneously presented. The oxidatively unstable *Nannochloropsis* suspension showed a faster carotenoid degradation as compared to the oxidatively stable *Phaeodactylum* suspension. For the bulk oils, it was even clearer: oxidative instability (for the 'Mixture 0.01' systems of both *Phaeodactylum* and *Tisochrysis*) was associated with a complete, (almost) immediate degradation of carotenoids, whereas the oxidatively stable 'Mixture 0.1' systems showed a gradual degradation towards non-zero values at the end of storage. Furthermore, the time point at which a complete carotenoid degradation could be observed also showed a clear primary oxidation peak: after 4 days for *Phaeodactylum* 'Mixture 0.01' and after 8 days for *Tisochrysis* 'Mixture 0.01'. The oxidatively unstable *Nannochloropsis* suspension of Chapter 3 on the other hand did not show a (visible) primary oxidation peak (as would be indicated by a ΔPV decrease) but also still contained carotenoids at the end of storage.

Concerning the *Phaeodactylum* suspensions of Chapter 4, where carotenoids were also analyzed throughout storage, making statements about their antioxidative role is more difficult since illuminated conditions were considered. Carotenoids can have a (physical) quenching role to prevent photo-oxidation without a degradation being observed [140]. Therefore, given their abundance throughout the whole storage experiment in this chapter, it can only be stated that the carotenoids **could have been working as a quencher**, but without certainty.

7.1.1.2. IMPACT OF QUANTITY

In exerting their antioxidative role, **the amount of carotenoids matters**. The presence of microalgal carotenoids on its own, like was suggested to be determinative for obtaining oxidative stability in former research [9], does not suffice. Again, this was observed **for suspensions as well as for bulk oils** throughout the different experimental chapters.

Chapter 3 illustrated the oxidative stability differences between *Phaeodactylum* and *Nannochloropsis* suspensions (respectively stable and unstable), both containing carotenoids but in a clearly different amount (initially threefold higher for *Phaeodactylum*). In Chapter 4, which further considered the *Phaeodactylum* suspensions but under light storage conditions, it was shown that illumination

only had a minor influence on the carotenoid content throughout storage. In that way, this did not jeopardize their presence in sufficient amounts to reassure oxidative stability.

With a switch in research matrix, Chapter 5 showed that the presence of microalgal carotenoids (more specifically of *Phaeodactylum*) in n-3 LC-PUFA rich bulk oil was also no guarantee for oxidative stability: the addition of 2.5% *Phaeodactylum* biomass to refined fish oil showed no oxidation inhibition at all in spite of a detectable carotenoid transfer. The impact of quantity in this was clearly shown in a following set-up in which mixtures of fish oil and *Phaeodactylum* total lipid extract were considered. It became clear that the relative amount of carotenoids (mainly consisting of fucoxanthin) to n-3 LC-PUFA was a good indicator for predicting oxidative stability. The generalizability of this finding was confirmed in Chapter 6 by also implementing *Tisochrysis* total lipid extracts, differing in lipid class composition and (slightly) in relative carotenoid composition. Combining the findings of both *Phaeodactylum* and *Tisochrysis* systems, it could be stated that bulk oil mixtures with a carotenoid/n-3 LC-PUFA ratio of 0.115 (±0.003) mg/mg led to oxidatively stable systems whereas mixtures with a ratio of 0.0099 (±0.0002) mg/mg showed thorough lipid oxidation. Furthermore, this last experimental chapter indicated the **importance of initial quantity**: if not abundant enough from the start, the carotenoids show a faster degradation than when preventing lipid oxidation, by simply oxidizing together with the lipids of which the autoxidation process could not be sufficiently inhibited.

Considering the focus on the carotenoid/n-3 LC-PUFA ratio for bulk oils in Chapter 6, it was interesting to investigate whether this had a similar declarative value in the aqueous biomass suspensions of Chapter 3. The calculated ratios were 0.30 (±0.02) mg carotenoids/mg n-3 LC-PUFA for *Phaeodactylum* and 0.131 (±0.006) mg carotenoids/mg n-3 LC-PUFA for *Nannochloropsis*. Since the latter was higher than the above mentioned 0.115 (±0.003) mg/mg, but still led to oxidative instability, it is clear that these values should not be adopted for suspensions. It shows an influence of system properties, a factor of which the impact cannot be underestimated (as was discussed in section 2.1.3.3. of the literature review), or carotenoid type (clearly differing between *Nannochloropsis* and *Phaeodactylum* or *Tisochrysis*). This impact is discussed further in section 7.1.1.3.

7.1.1.3. IMPACT OF TYPE AND SYSTEM

Unlike for the impact of quantity, **conclusions about the impact of carotenoid type are harder to make**. As was discussed in Chapter 3, looking at the carotenoid profile differences of *Phaeodactylum* and *Nannochloropsis* suspensions in the light of their oxidative stability differences, it is hard to state whether or not the antioxidative capacity of fucoxanthin (*Phaeodactylum*) is higher than the one of zeaxanthin or violaxanthin (*Nannochloropsis*) since their initial amounts were already different. Comparing different carotenoids within a certain system, on the other hand, is feasible. For this, evaluating the degradation rate constants obtained by first-order degradation modeling proved to be meaningful in Chapter 6 (section 6.3.3.2.). For the bulk oil systems of both *Phaeodactylum* and *Tisochrysis*, it could be observed that β-carotene had a higher tendency to act as an antioxidant compared to fucoxanthin or diatoxanthin, since its degradation rate was significantly higher.

Comparable to calculating the carotenoid/n-3 LC-PUFA ratio in the previous section in a search for supplemental insight, it was interesting to determine the degradation rate constants for the biomass suspensions of Chapter 3 as well. This was done by also applying a first-order degradation model on the different carotenoids throughout storage. Figure 7.2 shows a comparison of the first-order degradation rate constants of the biomass suspensions of Chapter 3 and both 'Mixture 0.1' bulk oil systems of Chapter 6. The 'Pure total lipid extract' and 'Mixture 0.2' systems were not presented since they only showed small differences with the 'Mixture 0.1' systems (6.3.3.2., Figure 6.6).

Considering these degradation rate constants, different observations can be made. First of all, when comparing the biomass suspensions, the degradation rate constants of the *Nannochloropsis* carotenoids were clearly higher, reflecting the faster degradation that was already discussed above (7.1.1.1.). Secondly, when comparing the *Phaeodactylum* carotenoids across different systems, it is clear that the degradation rate constants of both fucoxanthin and β-carotene were lower for the biomass suspension as compared to the bulk oil system. Both however showed to be oxidatively stable. This difference implies an impact of system properties, confirming the statement (touched upon in the literate review) that **extrapolating findings on antioxidative action** (of which the carotenoid breakdown has proven to be a reflection) **is not self-evident across different systems** [179].

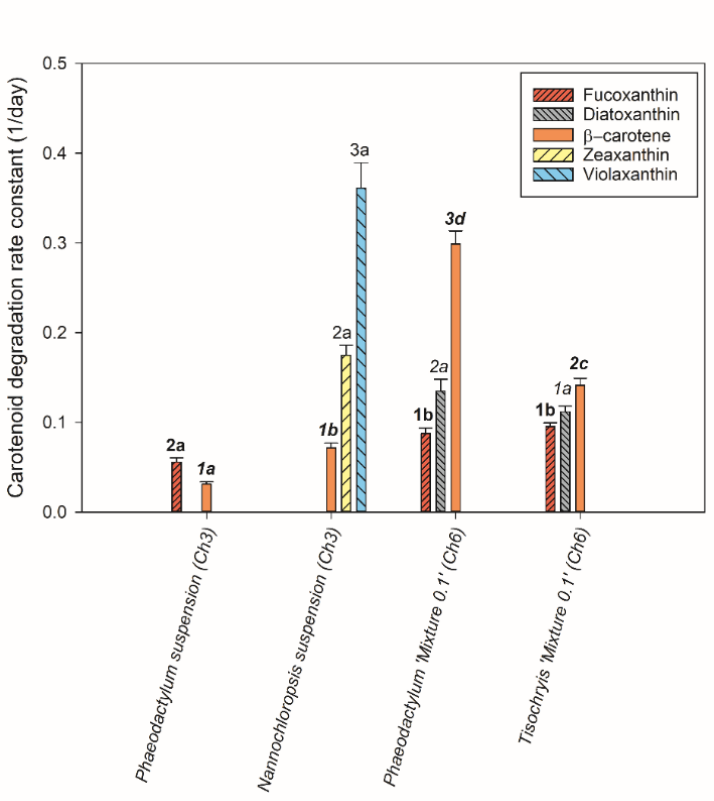


Figure 7.2: First-order degradation rate constants (in day^{-1}) of the *Phaeodactylum* and *Nannochloropsis* biomass suspensions of Chapter 3 and the *Phaeodactylum* and *Tisochrysis* 'Mixture 0.1' bulk oil systems of Chapter 6. Significant differences between different carotenoids are indicated by a different number within each system. Significant differences between different systems are indicated by a different letter for each carotenoid.

Another aspect that fits within the impact of system properties is the order difference of the carotenoids' degradation rate constants: whereas β-carotene was characterized by a significantly higher degradation rate constant as compared to the xanthophylls for the bulk oil systems, a significantly lower degradation rate constant as compared to the xanthophylls was observed for both biomass suspensions. Given the fact that carotenes are less polar than xanthophylls, this finding may suggest polarity effects. However, when the polar paradox (i.e. the most general attempt to explain antioxidant differences across different systems based on polarity as was explained in section 2.2.3.3.) is taken into account, the opposite findings are actually true: the more polar antioxidants were more active in the polar biomass suspensions, whereas the more apolar antioxidants were

more active in the apolar bulk oils. It once again shows that more complexity is at stake [179]. An additional aspect that could be of importance for the biomass suspensions is the localization of carotenoids within the microalgal cell. According to Gong & Bassi [275], the primary carotenoids are mostly located within the thylakoid membranes carrying out their photosynthesis assisting role, whereas the secondary carotenoids are accumulated within oily droplets to form a protective layer in stressed conditions. No specific information about which carotenoids are preferably located where in *Phaeodactylum* or *Nannochloropsis* was found. Possible differences in this location may already cause differences in proximity to the site of oxidation, explaining why polarity is not fully determinative. Additionally, cell integrity of the used biomasses and the impact of suspension preparation, might also have had its influence on the proximity of carotenoids.

7.1.1.4. MULTIPLE CAROTENOIDS MATTER

Whereas no one-sided conclusions could be made regarding the impact of carotenoid type or system, an additional conclusion that can be made in the light of the antioxidative importance of microalgal carotenoids is that **the role of different carotenoids should not be regarded separately**. This statement can easily be illustrated by considering β-carotene, a carotenoid that was present in all of the systems that were investigated throughout the different experimental chapters (with the exception of the pure fish oil systems). In Chapter 3, β-carotene was present in similar initial amounts for both suspensions, which did show a clearly different degree of degradation (with the breakdown in the oxidatively unstable *Nannochloropsis* suspension being more extensive). Also, at the end of storage, β-carotene completely degraded in most of the oxidatively stable bulk oil systems of Chapter 6, while for the oxidatively unstable *Nannochloropsis* suspension of Chapter 3, β-carotene was still present. Therefore, although the antioxidative role of β-carotene was clearly shown by its degradation throughout storage (7.1.1.1.), its presence or amount on its own had no declarative value in predicting oxidative outcome. By taking the other carotenoids into account, these apparent contradictory outcomes however could be explained. In Chapter 3, the presence of a sufficient amount of fucoxanthin throughout storage in the *Phaeodactylum* suspensions helped in ensuring oxidative stability and relieved 'pressure' on β-carotene as an antioxidant, while for the *Nannochloropsis* suspensions the other carotenoids quickly degraded, leaving β-carotene as the only remaining carotenoid with an antioxidative role. In Chapter 6, other

carotenoids were also still present to establish oxidative stability in the oxidatively stable bulk oil systems despite the full degradation of β-carotene.

7.1.2. THE IMPORTANCE OF MINOR EFFECTS

Although the major antioxidative importance of endogenous carotenoids was clearly shown by multiple aspects (degradation throughout storage, with an impact of quantity, in suspensions as well as in bulk oils, for all carotenoids, resulting in a clear link with oxidation), **minor effects cannot be excluded**. First of all, a minor influence of the other microalgal antioxidants, being phenolic compounds, ascorbic acid and tocopherols (see section 2.2.5.3.), cannot be excluded, especially since synergistic effects with carotenoids have been reported [172,177]. For the bulk oil systems of Chapters 5 and 6, an effect of ascorbic acid is however very unlikely since its high water-solubility most probably prevents it from being extracted during the water counter extracted total lipid extraction. Secondly, also for the lipid class composition (of which the contribution towards oxidative stability was estimated in Chapter 6), small amounts of polar lipids still being present in the *Tisochrysis* systems might have had an impact, albeit minor as well.

7.1.3. OXIDATIVE STABILITY ANALYSIS

Measuring lipid oxidation (or in other words the oxidative stability of a certain lipid rich system) was a common aspect of the experimental set-up of the different chapters of the current PhD research. As was touched upon in the literature review (2.1.4.1.), an accurate estimation of lipid oxidation progression requires multiple analyses at different time points. In the current research, this comprised the analysis of primary oxidation products (with a spectrophotometric FOX method), specific secondary oxidation products (with HS-SPME Arrow GC-MS) and the amount of n-3 LC-PUFA as most susceptible oxidation substrate (with GC-FID). Based on the obtained results, different conclusions could be drawn regarding good practices in using these methods for analyzing the oxidative stability of n-3 LC-PUFA rich products.

First of all, it could be observed that, when the **evolution of n-3 LC-PUFA content** was measured, it was **consistent with** the **analysis of primary oxidation** products (PV) throughout the whole PhD research. The appearance of primary oxidation was reflected in a decrease of n-3 LC-PUFA and vice versa. Moreover, the amount of n-3 LC-PUFA degradation was indicative of the degree of lipid oxidation. For the oxidatively unstable systems of Chapter 4 and 6 (i.e. the *Schizochytrium*

suspension of Chapter 4, and the 'Mixture 0.01', 'Mixture 0.001' and 'Pure Fish oil' systems of Chapter 6), in which a clear primary oxidation peak (indicating thorough lipid oxidation) could be measured, a complete (or almost complete) degradation of n-3 LC-PUFA could be observed. For the oxidatively unstable *Nannochloropsis* suspensions of Chapter 3, where the amount of primary oxidation products did not yet show an observable decrease, a partial n-3 LC-PUFA degradation was measured. The oxidatively stable systems (the *Phaeodactylum* suspensions of Chapter 3 and 4, and the 'Mixture 0.1', 'Mixture 0.2' and 'Pure Total lipid extract' systems of Chapter 6), showing only minor PV increases throughout storage, showed no or relatively small amounts of n-3 LC-PUFA degradation. Therefore, despite the fact that **analyzing fatty acids** is considered an insensitive method for estimating lipid oxidation [223], it has proven to be a **valuable additional analysis** for the n-3 LC-PUFA rich systems of the current PhD research.

As opposed to the consistency between n-3 LC-PUFA and primary oxidation analysis, consistency with the analysis of **secondary oxidation products** was not always present. Despite its sensitivity and specificity (in measuring a range of different volatile markers) it was **only of 'variable strength'**. This was clearly illustrated for the *Nannochloropsis* systems of Chapter 3, in which secondary oxidation markers (2-pentenal, 2,4-heptadienal and 2,6-nonadienal) were not at all indicating the occurrence of lipid oxidation while the opposite was proven by the other analyses. A possible explanation was sought in the (fast) further reaction of these aldehydes towards Strecker degradation products, leaving their presence unnoticed, like was previously described for krill oil studies [83]. Although the appearance of so-called 'Strecker aldehydes' was confirmed (also with GC-MS), the relation with lipid oxidation in microalgae requires further research. For the bulk oil mixtures of Chapter 6 on the other hand, the evolution of secondary oxidation products was completely in line with the ones of primary oxidation products and n-3 LC-PUFA. Despite this consistency contradiction between Chapters 3 and 6, analyzing the secondary oxidation products still proved to be of added value in the other research chapters. In Chapter 4, the illuminated *Phaeodactylum* suspensions showed an early increase of 2-butenal, indicating a certain appearance of photo-oxidation that, despite of its minor relevance compared to the thorough autoxidation in *Schizochytrium*, would have been left unnoticed with the analysis of only primary oxidation and n-3 LC-PUFA evolution. In Chapter 5, the analysis of secondary oxidation products seemed to confirm the

hypothesis for the underestimation of hydroperoxides that was encountered by the sampling method in the first part of the experimental set-up.

In can thus be concluded that, if feasible, **preferably all three analyses** should be performed **throughout storage**: the combination of primary oxidation and n-3 LC-PUFA content for making **accurate estimations** on oxidation progression, and the analysis of secondary oxidation products for giving **added value** by the analysis **of specific oxidation products**. None of the three analyses suffices on their own for claiming oxidative stability when no remarkable increases or decreases are measured. However, for getting a quick indication, n-3 LC-PUFA analysis is the least labor intensive one. Also, while primary and some secondary oxidation products can both increase and decrease throughout storage, possibly leaving them unnoticed in case of an unfortunate choice of time points, n-3 LC-PUFA can only decrease. Analyzing them could therefore be indicative of having 'missed' a primary or secondary 'oxidation peak'. Important to retouch upon is the fact that this only applies for 'lower' temperatures, since at higher ones oxidation kinetics differ (> 40 °C for high PUFA oils) [148] and n-3 LC-PUFA degradation could also be caused by thermal degradation (>180 °C) [216,217].

7.1.4. FUTURE FUNDAMENTAL ORIENTED PERSPECTIVES

For gaining further fundamental insight, a distinction can be made between resorting to 'simpler' systems or continuing to investigate similar ones.

As was touched upon in section 7.1.2., minor effects cannot be excluded when other (possibly antioxidative) compounds are present. Therefore, storage experiments of **simplified systems** with pure lipids and carotenoids (representative for the ones in microalgae) would be helpful in further gaining fundamental insight into the antioxidative capacities of microalgal carotenoids 'on their own'. However, this would result in extremely expensive experimental set-ups since it requires large amounts of pure compounds. In vitro assays on the other hand only require a small amount of sample but are of limited predictive value (as was discussed in 2.2.4.1.). If the high cost price would not be an obstacle, simplified systems would yield more focused conclusions towards the impact of carotenoid type by comparing systems with the same lipids but with different (pure) carotenoids. Also pure lipid classes could be used and compared in this to gain more insight into the (singular) impact of lipid class composition. In addition, adding different pure photosensitizers (relevant for microalgae) with a storage under illumination, could give additional fundamental insight in the process of photo-oxidation. Former research in that regard is limited and the

photosensitizing properties of pheophytin a and chlorophyll c as compared to chlorophyll a were a question of interest in Chapter 4 that could not be answered.

Although model systems with real microalgae or microalgal extracts contain much more compounds than only n-3 LC-PUFA and carotenoids, which makes drawing unequivocal conclusions more challenging, **further elaborating on similar systems** as the ones used in the current PhD research could still lead to additional fundamental insight. Open questions from Chapters 3, 4 and 6 could be a starting point.

Regarding the oxidative stability differences of the microalgae suspensions of **Chapter 3**, two supplemental approaches would be interesting towards the importance of endogenous carotenoids in ensuring n-3 LC-PUFA stability. On the one hand, a longer storage experiment of the *Phaeodactylum* suspensions with a parallel analysis of lipid oxidation and carotenoid profile, could indicate at which carotenoid content lipid oxidation can no longer be inhibited. Gheysen et al. [110] did report the oxidative stability of *Phaeodactylum* enriched aqueous model systems, containing a similar initial carotenoid profile, during 12 weeks of storage (37 °C). The carotenoid content throughout storage was however not investigated. On the other hand, to get more insight into the impact of carotenoid type vs. amount, a *Nannochloropsis* biomass leading to a similar initial carotenoid amount as for the (oxidatively stable) *Phaeodactylum* suspensions could be searched for. However this approach seems to be challenging since diverse test batches of commercially produced *Nannochloropsis* biomasses across Europe all showed lower carotenoid contents than the one used in the current experimental set-up.

For **Chapter 4**, the main question remaining was whether the absence of meaningful photo-oxidation under illuminated conditions was a consequence of the quenching capabilities of the carotenoids, the limited amount of photosensitization (due to fast chlorophyll breakdown and/or lower sensitizing properties of pheophytin), the low transparency of the investigated suspensions or a combination. Although combined effects are again difficult to exclude, some additional set-ups may provide additional information. First of all, suspensions with a neutral instead of acidic pH could be investigated since the low pH was probably the reason for the fast chlorophyll degradation [239]. Secondly, a higher light intensity or storage recipients resulting in a higher volume average light intensity may be thought of. Thirdly, other microalgae with a different pigment composition might be investigated although this does not necessarily result in stronger conclusions. The results were not implemented in the experimental chapters, yet the *Nannochloropsis* suspensions were also exposed to an illuminated storage experiment with a similar set-up as in Chapter 4. Just like for the *Phaeodactylum* suspensions, only minor differences could be measured

between a dark and light set-up regarding primary oxidation products, n-3 LC-PUFA content and pigment profile throughout storage. Therefore, possible photo-oxidation also did not result in a faster lipid oxidation appearance, with the same possible explanations applying.

With the clear separation in oxidative stability between the mixed bulk oil systems of **Chapter 6** (either extremely stable or extremely unstable) a follow-up experiment to investigate intermediate carotenoid/n-3 LC-PUFA ratio's is obvious. It would be interesting to investigate whether an intermediate ratio, between 0.0099 (±0.0002) and 0.115 (±0.003) mg/mg, would result in a more 'transitional' appearance of lipid oxidation rather than a clear-cut difference.

7.2. CONCLUSIONS TOWARDS PRACTICAL APPLICATIONS

The current PhD research was more fundamentally than practically oriented. Nevertheless, several insights led towards different points of attention for the development of n-3 LC-PUFA rich microalgae enriched food products or the use of microalgal compounds in obtaining oxidatively stable food products.

It should be noted that the statements on oxidative stability were based on storage experiments at 37 °C. Taking into account that every increase of 10 °C doubles the rate of the autoxidation process (i.e. a Q_{10} value of 2) [139,147], an autoxidative stability of 4 weeks at 37 °C translates into a stability of 13 weeks at room temperature (20 °C) and 40 weeks at refrigerated temperature (4 °C).

7.2.1. MICROALGAE AS ALTERNATIVE N-3 LC-PUFA SOURCE

First of all, when thinking of microalgae as an alternative n-3 LC-PUFA source to enrich food products with, the most important finding was that **photoautotrophic n-3 LC-PUFA rich microalgae are not necessarily oxidatively stable**. Although their oxidative stability was formerly cited as being a major advantage [9] and was taken into account when considering the different novel n-3 LC-PUFA sources (Chapter 1, Table 1.3), this characteristic should be nuanced. Photoautotrophic n-3 LC-PUFA rich microalgae can lead to oxidatively stable products, and are therefore still beneficial, but not all of them do. Chapter 3 showed that **the amount (and possibly type) of endogenous carotenoids on top of their presence is** an **important** oxidative stability determining factor to be taken into account as well. Therefore, for developing microalgae enriched food products, species and/or batches with a sufficient amount of carotenoids should be aimed for,

although the presence of antioxidants in the food matrix may help in stabilizing lipids which are not stable in model systems.

Secondly, whereas autoxidative stability seemed to be more of a problem for certain photoautotrophic microalgae than was first anticipated, the appearance of photo-oxidation due to the abundance of possible photosensitizers (chlorophyll) seemed to be less of a problem. Chapter 4 clearly showed that **illumination did not result in a loss of oxidative stability**. Also the supplemental experiments on *Nannochloropsis* suspensions that were touched upon in 7.1.4. did not show a supplemental or faster oxidation. Therefore, the impact of illumination with visible light should be less of a worry when thinking of food products stored in transparent containers. The potential of using autoxidatively stable photoautotrophic microalgae for developing stable n-3 LC-PUFA rich food products still holds under those storage conditions.

7.2.2. MICROALGAE AS ALTERNATIVE ANTIOXIDANT

Apart from using microalgae as an alternative n-3 LC-PUFA source, the possibilities of using oxidatively stable n-3 LC-PUFA rich microalgae (more specifically *Phaeodactylum*) as an alternative antioxidant for naturally unstable n-3 LC-PUFA rich sources (fish oil) were explicitly investigated in Chapter 5. It was clear that ***Phaeodactylum* biomass addition** had **no potential**, in whatever amount of addition. N-3 LC-PUFA oxidation in fish oil could however be inhibited by microalgal **carotenoids**, as was shown by evaluating the oxidative stability of 'fish oil - microalgal total lipid extract mixtures' in parallel with the carotenoid profile, for both *Phaeodactylum* and *Tisochrysis* (Chapter 6). Nevertheless, **high initial amounts are (again) required**. Momentarily making abstraction of the possible impact of carotenoid type, the observation that 0.115 (±0.003) mg carotenoids/mg n-3 LC-PUFA suffices for obtaining oxidative stability in bulk oils (Chapter 6), requires an addition of at least 2.88 (±0.08)% carotenoids in a 25% n-3 LC-PUFA fish oil. Adding 0.248 (±0.005)% on the other hand would not suffice. **From a food industry perspective these are high additions**. In fats and oils, the synthetic antioxidants BHT and BHA are allowed at a level of respectively 0.1 and 0.2% [276,277]. For tocopherols, with the exception of α-tocopherol in refined olive oil, *quantum satis* levels (i.e. as much as needed but not more) are authorized, yet typical reported use values in industry are 0.5% [278]. Reports on the authorized use of carotenoids as food additives are limited to the carotenoids β-carotene and lutein (albeit as a colorant for the latter), but also describe typical use ranges in ppm rather than in percentages [279,280]. However, it should be noted that these

reported values do not necessarily apply to all n-3 LC-PUFA rich, and therefore oxidatively very unstable, oils.

7.2.3. FUTURE APPLICATION ORIENTED PERSPECTIVES

It is self-evident that **further insights towards practical applications require more practically oriented research**. Firstly, if microalgae as an alternative n-3 LC-PUFA source for food enrichment are aimed for, this means experimental set-ups with real food products, rather than the aqueous model systems of Chapter 3 and 4 in the current PhD research. Real food matrices add complexity (either anti- or pro-oxidative, by compound, environmental or system properties), making clear (fundamental) conclusions even more challenging than was already addressed in 7.1.2. However, they would give certainty about the oxidative outcome of a certain food product. Nevertheless, it seems wise to opt for microalgae with sufficient carotenoids that are known to remain stable on their own. Secondly, if endogenous carotenoids as an alternative antioxidant for stabilizing n-3 LC-PUFA rich oils are aimed for, a suitable extraction technique would deserve major attention although research in this topic has already been conducted. In Chapter 5 and 6, chloroform:methanol 1:1 was opted for to get a total lipid extract, but this solvent is not food-grade. Also, implementing a comparison with conventional antioxidants (synthetic or natural) would improve the practical relevance. Alternatively, non-'n-3 LC-PUFA' rich microalgae with high carotenoid amounts could rather be aimed for since obtaining an equally high carotenoid/n-3 LC-PUFA amount is easier when no endogenous n-3 LC-PUFA are present in the extract.

Thinking about using photoautotrophic n-3 LC-PUFA rich microalgae (or their endogenous carotenoids) in future food products inevitably leads back to the difficulties they still face. **Three major challenges**, explaining why microalgae are mainly tapping niche markets nowadays, can be distinguished: production constraints, consumer acceptance and inhibitory regulations.

Firstly, like was addressed in the literature review (1.3.2.2.), the **production** of photoautotrophic microalgae is characterized by a difficult scale-up and a high energy intensity, which results in a high cost price. However, technological progress across the entire production chain is predicted to drive this down [14].

Secondly, regarding **consumer acceptance**, enrichment with microalgae will also influence other food quality aspects beyond oxidation (color, flavor, texture etc.). Given that color is inherently connected with the carotenoids and their structure (although other compounds also impact color), a color difference cannot be avoided if a sufficient amount of carotenoids is required for obtaining oxidative

stability. Therefore, if a practical application is further thought of, the general approach should be to find a suitable application where a color difference is not a problem. A possible problem of flavor is related to the European taste pattern not being familiar with algae flavors (much less than Asian countries for example). Growing acceptance by incorporation in (again) suitable food products, might alter these [281].

Thirdly, a bottleneck inhibiting microalgae from making a faster progression in food industry, can be found in the **legislation**. Since, for Europe, only *Arthrospira platensis* (former *Spirulina*), *Chlorella vulgaris*, *Chlorella pyrenoidosa*, *Chlorella luteoviridis* and *Aphanizomenon flos-aquae* were used prior to May 1997, other species need to be approved according to the Novel Food Regulation [282]. This goes along with a long and expensive application process. Among photoautotrophic microalgae, only the biomass of *Odontella aurita* and *Tetraselmis chui* and an astaxanthin-rich oleoresin of *Haematococcus pluvalis* have been approved so far [100]. Regarding the investigated species of the current PhD research, applications for *Nannochloropsis gaditana* and an EPA-rich oil of *Phaeodactylum tricornutum* have been cited, yet only for the latter a favourable opinion by the Food Safety Authority of Ireland has been presented so far [102,103,282]. A more dynamic and cheaper but safe revision of the legislation procedures, would enable promising species (like *Phaeodactylum* in the current research) to fulfil their potential [281].

Reflecting on the novel n-3 LC-PUFA sources that are needed to enlighten the pressure on the traditional ones, the above challenges make it clear that microalgae are only promising on longer term. This despite the clear potential (of some species) for obtaining oxidative stability. For **transgenic plants**, the infrastructure for large scale oilseed crop production, and consumption is already well-established, making them more promising on short term, while they also face regulatory issues regarding genetic modification (Chapter 1). Since information about their **oxidative stability** is however lacking, investigating this along with the carotenoid profile would be very interesting in the light of the current PhD research. Furthermore, investigating the oxidative stability of mixotrophic n-3 LC-PUFA rich microalgae would be interesting as well, since they possibly combine the advantages of endogenous antioxidative carotenoids (if abundant enough) and growth by fermentation giving rise to higher cell densities and, in that way, lower production costs [94].

APPENDIX

This appendix consists of a more extensive discussion on the **HS-SPME Arrow GC-MS** method that was implemented for measuring volatile secondary oxidation products in the current PhD research (section 3.2.2.5.). It gives a description of the HS-SPME GC-MS method that was used as a starting point, the method's optimization given the change to an Arrow fiber, and an illustration of the resulting method's suitability for following oxidation throughout storage. Finally, some points of attention for quantification were further touched upon.

A.1. STARTING POINT

As a starting point, the HS-SPME GC-MS method, with conventional SPME instead of SPME Arrow, of Van Durme et al. [283] can be referred to. This method for evaluating the volatile composition of different microalgae used a triple phase DVB/CAR/PDMS SPME fiber after comparison with PDMS and CAR/PDMS fibers. It enables the detection of a wide range of volatile compounds [165]. Following 30 min of sample incubation at 40 °C, an extraction time and temperature of respectively 30 min and 40 °C were opted for. Further used was a thermal desorption of 3 min at 250 °C with splitless injection on a non-polar cross-linked methyl silicone column (HP-PONA, 50 m x 0.20 mm, 0.5 µm film thickness) with helium as the carrier gas (1 mL/min). A temperature gradient from 40 °C (5 min) to 250 °C (5 min) increasing 5 °C/min was applied.

The method of Van Durme et al. [283] was also applied for analyzing secondary oxidation products in microalgae suspensions (PhD research of Lore Gheysen [9]) and microalgal as well as fish oil [81]. Both however used an incubation and extraction temperature of 60 °C instead of 40 °C, and an extraction time of 35 min instead of 30 min. Observing the successful use of this method (with slight modifications) for these oxidation studies, similar settings were started from for setting up the HS-SPME Arrow GC-MS method of the current PhD research. Extraction time and temperature, split ratio and required fiber desorption were however reconsidered, given the change of conventional SPME towards SPME Arrow fibers possessing a higher sorbent phase volume and robustness [169]. The triple DVB/CAR/PDMS phase, also being available for SPME Arrow, was not questioned again.

A.2. OPTIMIZATION ON ARROW FIBER

A.2.1. INVESTIGATED SYSTEM

Optimizing parameters required a system which is known to contain or form secondary oxidation products. For this, *Schizochytrium*, a heterotrophic, n-3 LC-PUFA rich microalga known for its fast oxidation [8], was selected. It was suspended in an aqueous system and stored for oxidation to occur (4 weeks, 37 °C). Marker volatiles that were reported in former research [8,9,110] were searched for using the NIST MS Search 2.3 spectral library. The following compounds were detected with reasonable identification accuracy (reverse match factor > 850): 2-butenal, 2-pentenal, hexanal, 2-heptenal, 2,4-heptadienal, 3,5-octadien-2-one, 2,6-nonadienal and 2,4-decadienal. Building a processing method for parameter optimization included a selection of suitable quantifying and confirming ions (QI and CI's) for each of these compounds based on their mass spectrum (Table A.1). The QI was used for quantification, the two CI's for identity confirmation.

Table A.1: Retention time (RT), quantifying ion (QI) and confirming ions (CI1 and CI2) of the detected volatile compounds

Compound	RT (min)	QI (m/z)	CI1 (m/z)	CI2 (m/z)
2-Butenal	6.1	41	70	69
2-Pentenal	9.5	83	55	84
Hexanal	11.5	44	56	72
2-Heptenal	17.4	41	70	55
2,4-Heptadienal	18.8	81	110	53
3,5-Octadien-2-one	21.4	124	95	43
2,6-nonadienal	24.1	70	41	69
2,4-Decadienal	28.4	81	152	67

The retention time (RT) was confirmed by the use of external standards. Only for 3,5-octadien-2-one no standard could be found. Due to their specificity for n-3 LC-PUFA oxidation (as was addressed in the literature review, section 2.1.4.3.) and external standard confirmation, 2-pentenal, 2,4-heptadienal and 2,6-nonadienal were given most attention.

By way of illustration, Figure A.1 shows the total ion chromatogram (TIC) for an oxidized *Schizochytrium* suspension and the resulting single ion chromatogram (SIC), i.e. for a certain window with a different QI for each of the compounds when applying the obtained processing method. Figure A.2 illustrates the use of CI's (for 2,4-heptadienal as an example), showing peaks at the same retention time as the QI. It also shows the obtained mass spectrum (at the RT in question) and the mass spectrum available in the library.

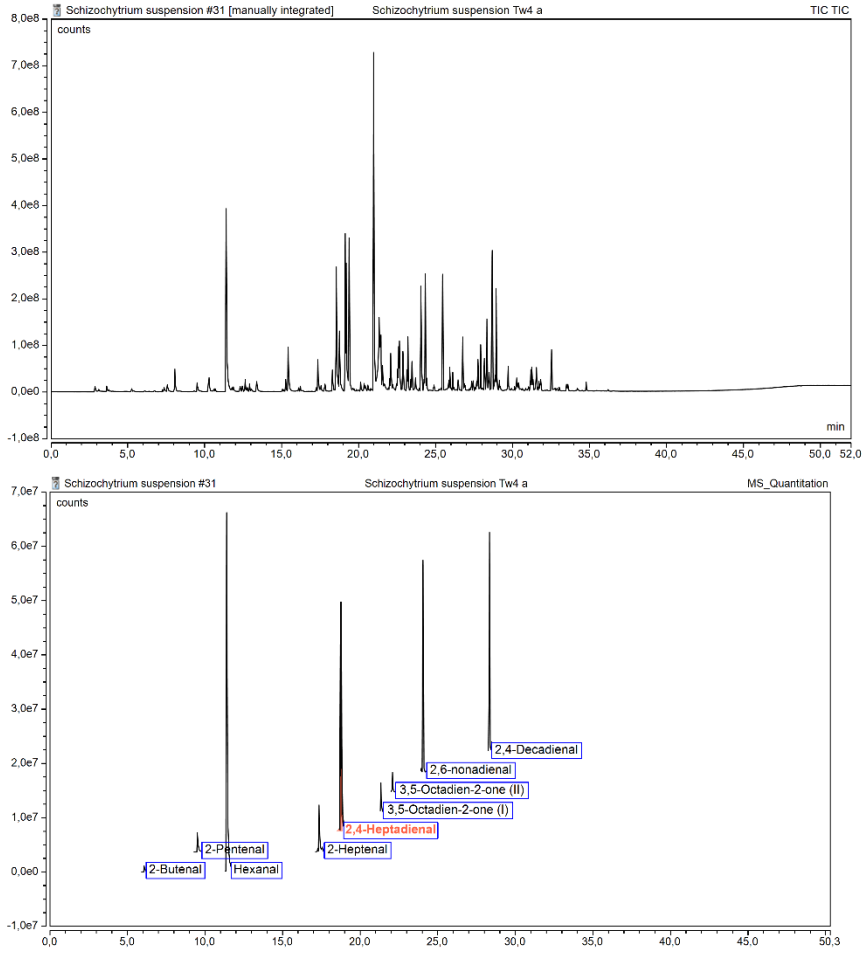


Figure A.1: Total ion chromatogram (TIC) and processed single ion chromatogram (SIC) of an oxidized *Schizochytrium* suspension

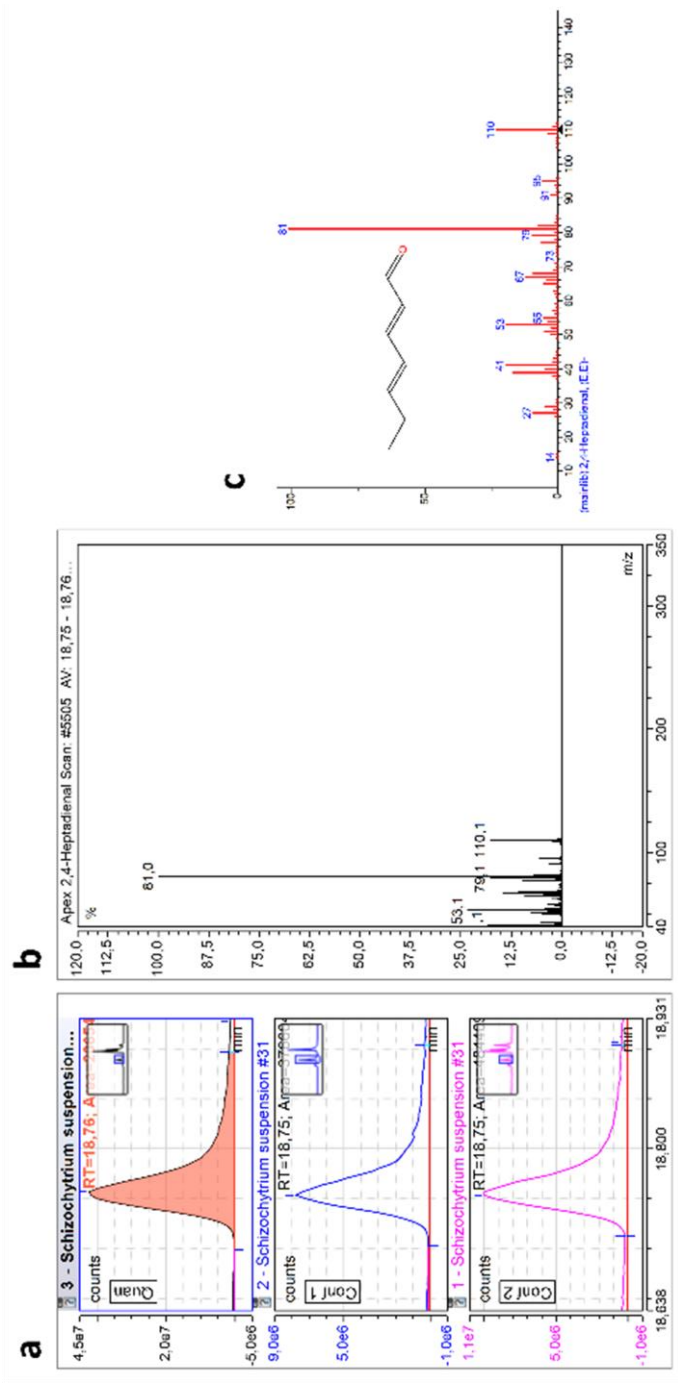


Figure A.2: Concept of quantifying ion (QI) and confirming ions (CI1 and CI2) for 2,4-heptadienal (a) together with the observed mass spectrum (b) and the library spectrum of 2,4-heptadienal (c)

A.2.2. PARAMETERS

Using the above described system and processing method, four parameters were investigated: fiber postdesorption time, split injection ratio, incubation and extraction temperature and extraction time. When comparing different settings, the SIC peak areas were used without further conversion, since relative differences were sufficient for this purpose.

For analyzing the volatile oxidation products of different samples, it is important that compounds from one sample are completely desorbed before adsorbing the compounds of a subsequent sample. Therefore, after desorption on the column, a subsequent postdesorption at 270 °C (in a separate station) was implemented to remove the remaining compounds. A **postdesorption time** of 5 min proved to be sufficient, as indicated by a clean fiber blank after sample analysis of a thoroughly oxidized sample (*Schizochytrium* suspension, 4 weeks, 37 °C).

Due to the higher sorbent phase volume of the SPME Arrow fiber, splitless injection led to peak intensities that were too high for proper analysis (> 1E9 in the TIC). Therefore, a **split injection** was applied. Both ratios of 1/25 and 1/50 were tested, and the latter was opted for since it still led to intensities that were clearly high enough (ca. 1E8).

Based on the **incubation** and **extraction temperature** settings of the former methods (section A.1.), 40 °C and 60 °C were compared. For all of the compounds of interest, a temperature of 60 °C led to an equally large or larger peak area. Therefore, just like for the oxidation experiments of Gheysen et al. [110] and Ryckebosch et al. [81] an incubation and extraction temperature of 60 °C rather than 40 °C was selected.

Incubation and extraction times were lowered: with an incubation time fixed at 15 min, **extraction time**s of 5, 15 and 25 min were compared. Sampling time should reach the equilibrium point that is set between the sample and the fiber. Since 15 min of extraction mainly led to comparable results as 25 min of extraction, while 5 min led to lower peak areas for some compounds, 15 min was selected.

For the sake of completeness, it should be mentioned that the extraction time was reconsidered for investigating **oil systems** (an oxidized fish oil was used as a system of investigation rather than an oxidized *Schizochytrium* suspension). An extraction time of 15 min was again opted for. The sufficiency of a 5 min postdesorption was also reconsidered and confirmed. The split ratio of 1/50 and

the incubation and extraction temperature of 60 °C were not, since comparable intensities were observed and this temperature did not lead to undesired compound breakdown for the suspensions.

A.3. OXIDIZING SYSTEMS THROUGHOUT STORAGE

To estimate whether the obtained method was capable of detecting a systematic increase of secondary oxidation products throughout storage, a trial storage experiment (37 °C) of both *Schizochytrium* suspension and fish oil was set up with the analysis of secondary oxidation products on multiple time points. Figure A.3 shows the evolution of 2-pentenal and 2,4-heptadienal. It proves that the method can detect an increase towards a secondary oxidation peak for both type of systems.

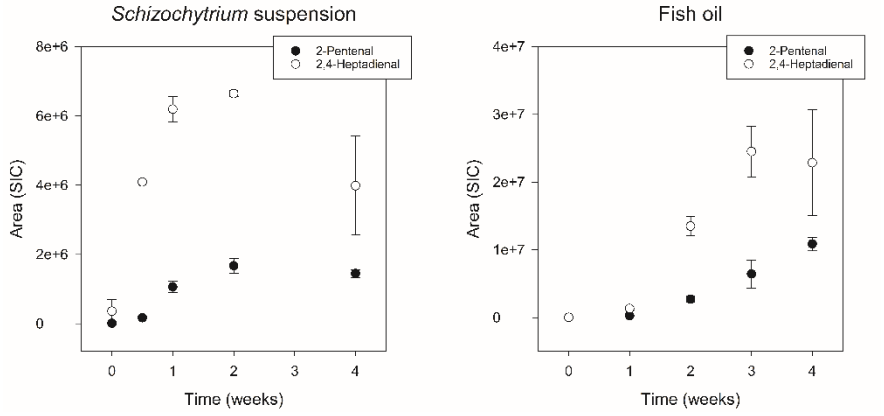


Figure A.3: 2-Pentenal and 2,4-Heptadienal evolution for *Schizochytrium* suspension and fish oil trial storage experiments in SIC peak area.

A.4. QUANTIFICATION

The SIC peak area of the different compounds is (amongst others) dependent on the relative abundance of the QI of choice. Therefore, the SIC peak areas still had to be converted to actual concentrations (in µg/100 g for the suspensions and in µg/mg for the oil) by the use of external calibration plots. This was performed for each of the compounds of which a standard was available. A point of attention when doing so, was that the (different concentrations of) standards were spiked into the matrix of interest. For Chapters 3 and 4, acidified aqueous systems (pH 4) were used. For Chapter 5 and 6, the standards were spiked into (unstored) oil samples. While for the aqueous suspensions a similar compound release as for the actual samples could be expected, this was not the case for the oil systems

since a high viscosity might counteract a proper dissolution of the spiked standards. This could result in easier compound release compared to the samples, but cannot be avoided since preparation of the oil samples in quantities of 10 mg/vial consists of a nitrogen flushing step which would blow away the standards when dissolving them beforehand. This explains the reported semi-quantitative properties of the method.

Another point of attention is the intensity variation in the MS signal. Especially comparing samples of different sequences should be done with care since the signal intensity might have been altered in the meantime. In other words, differences in peak areas and resulting concentrations could be due to sample differences as well as variations of signal intensities. To overcome this problem, peak areas were initially corrected by a 'tuning factor'. This factor was a relative expression of the intensity of one of the calibration peaks (m/z 69), and was determined before every sequence. Calibration peak m/z 69 was used rather than the other ones (m/z 219 or m/z 502) since it leaned closest to the QI's of the different compounds. This correction was applied for the results of Chapters 3, 4 and 5 (part A), and explained the semi-quantitative properties of the results. Hereafter, a better solution was implemented with the use of an internal standard (hexanal d-12) that did not coincide with the compounds of interest. In this way, each sample rather than each sequence could be corrected for intensity variations, which was the case for the results of Chapters 5 (part B) and 6.

REFERENCES

(1) Calder, P. C. Health Benefits of Omega-3 Fatty Acids. In *Omega-3 Delivery Systems*; García-Moreno, P. J., Jacobsen, C., Sorensen, A.-D. M., Yesiltas, B., Eds.; Academic Press, **2021**; pp 25–53.

(2) Naylor, R. L.; Goldburg, R. J.; Primavera, J. H.; Kautsky, N.; Beveridge, M. C. M.; Clay, J.; Folke, C.; Lubchenco, J.; Mooney, H.; Troell, M. Effect of Aquaculture on World Fish Supplies. *Nature* **2000**, *405* (6790), 1017–1024.

(3) FAO. *The State of World Fisheries and Aquaculture 2022*; FAO: Rome, **2022**.

(4) Tocher, D. R. Omega-3 Long-Chain Polyunsaturated Fatty Acids and Aquaculture in Perspective. *Aquaculture* **2015**, *449*, 94–107.

(5) Ryckebosch, E. Microalgae as Alternative Source of Long Chain Omega-3 Polyunsaturated Fatty Acids, KU Leuven, **2013**.

(6) Barta, D. G.; Coman, V.; Vodnar, D. C. Microalgae as Sources of Omega-3 Polyunsaturated Fatty Acids: Biotechnological Aspects. *Algal Res.* **2021**, *58* (102410).

(7) Frankel, E. N. *Lipid Oxidation: Second Edition*; Oily Press: Bridgewater, U.K., **2005**.

(8) Gheysen, L.; Demets, R.; Devaere, J.; Bernaerts, T.; Goos, P.; Van Loey, A.; De Cooman, L.; Foubert, I. Impact of Microalgal Species on the Oxidative Stability of N-3 LC-PUFA Enriched Tomato Puree. *Algal Res.* **2019**, *40* (101502).

(9) Gheysen, L. Oxidative Stability of Omega-3 Long Chain Poly-Unsaturated Fatty Acids in Vegetable Purees Enriched with Microalgae, KU Leuven, **2019**.

(10) Micha, R.; Khatibzadeh, S.; Shi, P.; Fahimi, S.; Lim, S.; Andrews, K. G.; Engell, R. E.; Powles, J.; Ezzati, M.; Mozaffarian, D. Global, Regional, and National Consumption Levels of Dietary Fats and Oils in 1990 and 2010: A Systematic Analysis Including 266 Country-Specific Nutrition Surveys. *BMJ* **2014**, *348* (April), 1–20.

(11) Gladyshev, M. I.; Sushchik, N. N.; Makhutova, O. N. Production of EPA and DHA in Aquatic Ecosystems and Their Transfer to the Land. *Prostaglandins Other Lipid Mediat.* **2013**, *107*, 117–126.

(12) Gheysen, L.; Matton, V.; Foubert, I. Microalgae as a Source of Omega-3 Polyunsaturated Fatty Acids. In *Polyunsaturated fatty acids (PUFAs): Food sources, health effects and significance in biochemistry*; Catala, A., Ed.; Nova Science Publishers: New York, **2018**; pp 1–40.

(13) Chacón-Lee, T. L.; González-Mariño, G. E. Microalgae for "Healthy" Foods-Possibilities and Challenges. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9* (6), 655–675.

(14) Ruiz, J.; Olivieri, G.; De Vree, J.; Bosma, R.; Willems, P.; Reith, J. H.; Eppink, M. H. M.; Kleinegris, D. M. M.; Wijffels, R. H.; Barbosa, M. J. Towards Industrial Products from Microalgae. *Energy Environ. Sci.* **2016**, *9* (10), 3036–3043.

(15) Shahidi, F.; Ambigaipalan, P. Antioxidants in Oxidation Control. In *Measurement of Antioxidant Activity & Capacity*; Apak, R., Capanoglu, E., Shahidi, F., Eds.; John Wiley & Sons, Ltd: Chichester, UK, **2017**; pp 287–320.

(16) Hrebień-Filisińska, A. Application of Natural Antioxidants in the Oxidative Stabilization of Fish Oils: A Mini-Review. *J. Food Process. Preserv.* **2021**, *45* (4), 1–12.

(17) Tocher, D. R.; Betancor, M. B.; Sprague, M.; Olsen, R. E.; Napier, J. A. Omega-3 Long-Chain Polyunsaturated Fatty Acids, EPA and DHA: Bridging the Gap between Supply and Demand. *Nutrients* **2019**, *11* (1), 1–20.

(18) Calder, P. C. Very Long-Chain n-3 Fatty Acids and Human Health: Fact, Fiction and the Future. *Proc. Nutr. Soc.* **2018**, *77* (1), 52–72.

(19) Calder, P. C. Very Long Chain Omega-3 (n-3) Fatty Acids and Human Health. *Eur. J. Lipid Sci. Technol.* **2014**, *116* (10), 1280–1300.

(20) Aglago, E. K.; Huybrechts, I.; Murphy, N.; Casagrande, C.; Nicolas, G.; Pischon, T.; Fedirko, V.; Severi, G.; Boutron-Ruault, M. C.; Fournier, A.; Katzke, V.; Kühn, T.; Olsen, A.; Tjønneland, A.; Dahm, C. C.; Overvad, K.; Lasheras, C.; Agudo, A.; Sánchez, M. J.; Amiano, P.; Huerta, J. M.; Ardanaz, E.; Perez-Cornago, A.; Trichopoulou, A.; Karakatsani, A.; Martimianaki, G.; Palli, D.; Pala, V.; Tumino, R.; Naccarati, A.; Panico, S.; Bueno-de-Mesquita, B.; May, A.; Derksen, J. W. G.; Hellstrand, S.; Ohlsson, B.; Wennberg, M.; Van Guelpen, B.; Skeie, G.; Brustad, M.; Weiderpass, E.; Cross, A. J.; Ward, H.; Riboli, E.; Norat, T.; Chajes, V.; Gunter, M. J. Consumption of Fish and Long-Chain n-3 Polyunsaturated Fatty Acids Is Associated With Reduced Risk of Colorectal Cancer in a Large European Cohort. *Clin. Gastroenterol. Hepatol.* **2020**, *18* (3), 654–666.

(21) Murphy, R. A.; Mourtzakis, M.; Chu, Q. S. C.; Baracos, V. E. Supplementation With Fish Oil Increases First-Line Chemotherapy Efficacy in Patients With Advanced Nonsmall Cell Lung Cancer. **2011**, 3774–3780.

(22) Hashimoto, M.; Hossain, S.; Al Mamun, A.; Matsuzaki, K.; Arai, H. Docosahexaenoic Acid: One Molecule Diverse Functions. *Crit. Rev. Biotechnol.* **2017**, *37* (5), 579–597.

(23) Makrides, M.; Neumann, M. A.; Byard, R. W.; Simmer, K.; Gibson, R. A. Fatty Acid Composition of Brain, Retina, and Erythrocytes in Breast- and Formula-Fed Infants. *Am. J. Clin. Nutr.* **1994**, *60* (2), 189–194.

(24) Carlson, S. E. Docosahexaenoic Acid Supplementation in Pregnancy and Lactation. *Am. J. Clin. Nutr.* **2009**, *89* (2), S678–S684.

(25) Monroig, Ó.; Kabeya, N. Desaturases and Elongases Involved in Polyunsaturated Fatty Acid Biosynthesis in Aquatic Invertebrates: A Comprehensive Review. *Fish. Sci.* **2018**, *84* (6), 911–928.

(26) Castro, L. F. C.; Tocher, D. R.; Monroig, O. Long-Chain Polyunsaturated Fatty Acid Biosynthesis in Chordates: Insights into the Evolution of Fads and Elovl Gene Repertoire. *Prog. Lipid Res.* **2016**, *62*, 25–40.

(27) Marventano, S.; Kolacz, P.; Castellano, S.; Galvano, F.; Buscemi, S.; Mistretta, A.; Grosso, G. A Review of Recent Evidence in Human Studies of N-3 and n-6 PUFA Intake on Cardiovascular Disease, Cancer, and Depressive Disorders: Does the Ratio Really Matter? *Int. J. Food Sci. Nutr.* **2015**, *66* (6), 611–622.

(28) Plourde, M.; Cunnane, S. C. Extremely Limited Synthesis of Long Chain Polyunsaturates in Adults: Implications for Their Dietary Essentiality and Use as Supplements. *Appl. Physiol. Nutr. Metab.* **2007**, *32* (4), 619–634.

(29) Burdge, G. α-Linolenic Acid Metabolism in Men and Women: Nutritional and Biological Implications. *Curr. Opin. Clin. Nutr. Metab. Care* **2004**, *7* (2), 137–144.

(30) Horrobin, D. F. Loss of Delta-6-Desaturase Activity as a Key Factor in Aging. *Med. Hypotheses* **1981**, *7* (9), 1211–1220.

(31) Cunnane, S. C. Role of Zinc in Lipid and Fatty Acid Metabolism and in Membranes. *Prog. Food Nutr. Sci.* **1988**, *12* (2), 151–188.

(32) Mahaffey, K. R.; Sunderland, E. M.; Chan, H. M.; Choi, A. L.; Grandjean, P.; Mariën, K.; Oken, E.; Sakamoto, M.; Schoeny, R.; Weihe, P.; Yan, C. H.; Yasutake, A. Balancing the Benefits of N-3 Polyunsaturated Fatty Acids and the Risks of Methylmercury Exposure from Fish Consumption. *Nutr. Rev.* **2011**, *69* (9), 493–508.

(33) Simopoulos, A. P. The Omega-6/Omega-3 Fatty Acid Ratio: Health Implications. *Oléagineux, Corps gras, Lipides* **2010**, *17* (5), 267–275.

(34) Brenna, J. T.; Salem, N.; Sinclair, A. J.; Cunnane, S. C. α-Linolenic Acid Supplementation and Conversion to n-3 Long-Chain Polyunsaturated Fatty Acids in Humans. *Prostaglandins Leukot. Essent. Fat. Acids* **2009**, *80* (2–3), 85–91.

(35) Baker, E. J.; Miles, E. A.; Burdge, G. C.; Yaqoob, P.; Calder, P. C. Metabolism and Functional Effects of Plant-Derived Omega-3 Fatty Acids in Humans. *Prog. Lipid Res.* **2016**, *64*, 30–56.

(36) Stark, K. D.; Van Elswyk, M. E.; Higgins, M. R.; Weatherford, C. A.; Salem, N. Global Survey of the Omega-3 Fatty Acids, Docosahexaenoic Acid and Eicosapentaenoic Acid in the Blood Stream of Healthy Adults. *Prog. Lipid Res.* **2016**, *63*, 132–152.

(37) Adarme-Vega, T. C.; Thomas-Hall, S. R.; Schenk, P. M. Towards Sustainable Sources for Omega-3 Fatty Acids Production. *Curr. Opin. Biotechnol.* **2014**, *26*, 14–18.

(38) De Roos, B.; Sneddon, A. A.; Sprague, M.; Horgan, G. W.; Brouwer, I. A. The Potential Impact of Compositional Changes in Farmed Fish on Its Health-Giving Properties: Is It Time to Reconsider Current Dietary Recommendations? *Public Health Nutr.* **2017**, *20* (11), 2042–2049.

(39) Gladyshev, M. I.; Sushchik, N. N.; Tolomeev, A. P.; Dgebuadze, Y. Y. Meta-Analysis of Factors Associated with Omega-3 Fatty Acid Contents of Wild Fish. *Rev. Fish Biol. Fish.* **2018**, *28* (2), 277–299.

(40) Puri, M.; Thyagarajan, T.; Gupta, A.; Barrow, C. J. Omega-3 Fatty Acids Produced from Microalgae. In *Springer Handbook of Marine Biotechnology*; Kim, S.-K., Ed.; Springer: Berlin, **2015**; pp 1043–1057.

(41) Arts, M. T.; Palmer, M. E.; Skiftesvik, A. B.; Jokinen, I. E.; Browman, H. I. UVB Radiation Variably Affects N-3 Fatty Acids but Elevated Temperature Reduces n-3 Fatty Acids in Juvenile Atlantic Salmon (Salmo Salar). *Lipids* **2012**, *47* (12), 1181–1192.

(42) Tacon, A. G. J.; Metian, M. Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply. *Rev. Fish. Sci.* **2013**, *21* (1), 22–38.

(43) Czerner, M.; Agustinelli, S. P.; Guccione, S.; Yeannes, M. I. Effect of Different Preservation Processes on Chemical Composition and Fatty Acid Profile of Anchovy (Engraulis Anchoita). *Int. J. Food Sci. Nutr.* **2015**, *66* (8), 887–894.

(44) FAO. *The State of World Fisheries and Aquaculture 2020*; FAO: Rome, **2020**.

(45) Link, J. S.; Watson, R. A. Global Ecosystem Overfishing: Clear Delineation within Real Limits to Production. *Sci. Adv.* **2019**, *5* (6), 1–12.

(46) Rizzo, G.; Baroni, L. Health and Ecological Implications of Fish Consumption: A Deeper Insight. *Med. J. Nutrition Metab.* **2016**, *9* (1), 7–22.

(47) Ahmed, N.; Thompson, S. The Blue Dimensions of Aquaculture: A Global Synthesis. *Sci. Total Environ.* **2019**, *652*, 851–861.

(48) Tacon, A. G. J.; Metian, M. Global Overview on the Use of Fish Meal and Fish Oil in Industrially Compounded Aquafeeds: Trends and Future Prospects. *Aquaculture* **2008**, *285* (1–4), 146–158.

(49) Kok, B.; Malcorps, W.; Tlusty, M. F.; Eltholth, M. M.; Auchterlonie, N. A.; Little, D. C.; Harmsen, R.; Newton, R. W.; Davies, S. J. Fish as Feed: Using Economic Allocation to Quantify the Fish In : Fish Out Ratio of Major Fed Aquaculture Species. *Aquaculture* **2020**, *528* (May), 735474.

(50) Sprague, M.; Dick, J. R.; Tocher, D. R. Impact of Sustainable Feeds on Omega-3 Long-Chain Fatty Acid Levels in Farmed Atlantic Salmon, 2006–2015. *Sci. Rep.* **2016**, *6* (21892).

(51) Knutsen, H. R.; Johnsen, I. H.; Keizer, S.; Sørensen, M.; Roques, J. A. C.; Hedén, I.; Sundell, K.; Hagen. Fish Welfare, Fast Muscle Cellularity, Fatty Acid and Body-Composition of Juvenile Spotted Wolffish (Anarhichas Minor) Fed a Combination of Plant Proteins and Microalgae (Nannochloropsis Oceanica). *Aquaculture* **2019**, *506* (February), 212–223.

(52) Flick, G. J.; Hong, G.; Knobl, G. M. Lipid Oxidation of Seafood During Storage. In *Lipid oxidation in Food*; St. Angelo, A., Ed.; American Chemical Society, **1992**; pp 183–207.

(53) Neff, M. R.; Bhavsar, S. P.; Braekevelt, E.; Arts, M. T. Effects of Different Cooking Methods on Fatty Acid Profiles in Four Freshwater Fishes from the Laurentian Great Lakes Region. *Food Chem.* **2014**, *164*, 544–550.

(54) Bakar, J.; Rahimabadi, E. Z.; Che Man, Y. B. Lipid Characteristics in Cooked, Chill-Reheated Fillets of Indo-Pacific King Mackerel (Scomberomorous Guttatus). *LWT - Food Sci. Technol.* **2008**, *41* (10), 2144–2150.

(55) Stephen, N. M. Effect of Different Types of Heat Processing on Chemical Changes in Tuna. **2010**, *47* (April), 174–181.

(56) Olsen, R. L.; Hasan, M. R. A Limited Supply of Fishmeal : Impact on Future Increases in Global Aquaculture. *Trends Food Sci. Technol.* **2012**, *27* (2), 120–128.

(57) Rubio-Rodríguez, N.; Beltrán, S.; Jaime, I.; de Diego, S. M.; Sanz, M. T.; Carballido, J. R. Production of Omega-3 Polyunsaturated Fatty Acid Concentrates: A Review. *Innov. Food Sci. Emerg. Technol.* **2010**, *11* (1), 1–12.

(58) Kouba, M.; Mourot, J. A Review of Nutritional Effects on Fat Composition of Animal Products with Special Emphasis on N-3 Polyunsaturated Fatty Acids. *Biochimie* **2011**, *93*, 13–17.

(59) Pike, I. H. Fish Oil: Supply and Demand as a Source of Long-chain N-3 Polyunsaturated Fatty Acids in the Human Diet. *Eur. J. Lipid Sci. Technol.* **2015**, *117* (6), 747–750.

(60) BioMar. *Sustainability Report 2019*; **2019**.

(61) Auchterlonie, N. A. *The continuing importance of fishmeal and fish oil in aquafeeds Presented at the Aquafarm Conference, Pordenone, Italy, 15-16 February 2018*. IFFO.

(62) Kulås, E. Extraction, Refining, Concentration, and Stabilization of Long-Chain Omega-3 Oils. In *Omega-3 Delivery Systems*; García-Moreno, P. J., Jacobsen, C., Sorensen, A.-D. M., Yesiltas, B., Eds.; Academic Press, **2021**; pp 55–78.

(63) Kazuo, M. Prevention of Fish Oil Oxidation. *J. Oleo Sci.* **2019**, *68* (1), 1–11.

(64) Knutsen, H. K.; Alexander, J.; Barregård, L.; Bignami, M.; Brüschweiler, B.; Ceccatelli, S.; Cottrill, B.; Dinovi, M.; Edler, L.; Grasl-Kraupp, B.; Hoogenboom, L. R.; Nebbia, C. S.; Oswald, I. P.; Petersen, A.; Rose, M.; Roudot, A.; Schwerdtle, T.; Vleminckx, C.; Vollmer, G.; Wallace, H.; Lundebye, A.; Metzler, M.; Colombo, P.; Hogstrand, C. Assessment of Decontamination Processes for Dioxins and Dioxin-like PCBs in Fish Oil by Physical Filtration with Activated Carbon. *EFSA J.* **2017**, *15* (12).

(65) Fernández-González, R.; Yebra-Pimentel, I.; Martínez-Carballo, E.; Simal-Gándara, J. Decontamination Solutions for Polychlorinated Biphenyls (PCBs) in Raw Fish Oils from Environmentally Contaminated Sea Fishes. *Sci. Total Environ.* **2014**, *468–469*, 1007–1013.

(66) FAO. *The State of World Fisheries and Aquaculture 2016*; FAO: Rome, **2016**.

(67) Olsen, R.-E.; Waagbo, R.; Melle, W.; Ringo, E.; Lall, S. P. Alternative Marine Resources. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*; Turchini, G. M., Ng, W.-K., Tocher, D. R., Eds.; CRC Press, Taylor & Francis Group: Boca Raton, **2011**; pp 267–324.

(68) Kattner, G.; Krause, M. Seasonal Variations of Lipids (Wax Esters , Fatty Acids and Alcohols) in Calanoid Copepods from The. *Mar. Chem.* **1989**, *26*, 261–275.

(69) Phleger, C. F.; Nichols, P. D.; Virtue, P. Lipids and Trophodynamics of Antarctic Zooplankton. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **1998**, *120*, 311–323.

(70) Alvheim, A. R.; Kjellevold, M.; Strand, E.; Sanden, M.; Wiech, M. Mesopelagic Species and Their Potential Contribution to Food and Feed Security—a Case Study from Norway. *Foods* **2020**, *9* (3), 1–19.

(71) Uddin, S.; Kishimura, H.; Chun, B. Isolation and Characterization of Lecithin from Squid (Todarodes Pacificus) Viscera Deoiled by Supercritical Carbon Dioxide Extraction. *J. Food Sci.* **2011**, *76* (2), 350–354.

(72) Park, J.; Lee, H.; Lee, G.-Y.; Kim, H.-R.; Kim, Y.; Lee, Y. Development of Refined Cuttlefish (Todarodes Pacificus) Oil and Its Use as Functional Ingredients. *Food Sci. Biotechnol.* **2011**, *20* (2), 389–394.

(73) Ryckebosch, E.; Bruneel, C.; Muylaert, K.; Foubert, I. Microalgae as an Alternative Source of Omega-3 Long Chain Polyunsaturated Fatty Acids. *Lipid Technol.* **2012**, *24* (6), 128–130.

(74) Xie, D.; Gong, M.; Wei, W.; Jin, J.; Wang, X.; Wang, X.; Jin, Q. Antarctic Krill (Euphausia Superba) Oil: A Comprehensive Review of Chemical Composition, Extraction Technologies, Health Benefits, and Current Applications. *Compr. Rev. Food Sci. Food Saf.* **2019**, *18* (2), 514–534.

(75) Nicol, S.; Foster, J. The Fishery for Antarctic Krill: Its Current Status and Management Regime. In *Biology and Ecology of Antarctic Krill*; Siegel, V., Ed.; Springer International Publishing, **2016**; pp 387–421.

(76) European Commission. Commission Implementing Decision (EU) 2016/598 of 14 April 2016 Authorising an Extension of Use of Lipid Extract from Antarctic Krill (Euphausia Superba) as a Novel Food Ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Coun. *Off. J. Eur. Union* **2016**, No. 103, 34–36.

(77) Bucchini, L. Nutrition and Health Claims in Europe: Oils & Fats Related Claims, Regulatory and Labeling Challenges. *OCL - Oilseeds fats, Crop. Lipids* **2019**, *26*.

(78) Hill, S. L.; Murphy, E. J.; Reid, K.; Trathan, P. N.; Constable, A. J. Modelling Southern Ocean Ecosystems: Krill, the Food-Web, and the Impacts of Harvesting. *Biol. Rev. Camb. Philos. Soc.* **2006**, *81* (4), 581–608.

(79) Ulven, S. M.; Holven, K. B. Comparison of Bioavailability of Krill Oil versus Fish Oil and Health Effect. *Vasc. Health Risk Manag.* **2015**, *11*, 511–524.

(80) Bogevik, A. S.; Tocher, D. R.; Langmyhr, E.; Waagbø, R.; Olsen, R. E. Atlantic Salmon (Salmo Salar) Postsmolts Adapt Lipid Digestion According to Elevated Dietary Wax Esters from Calanus Finmarchicus. *Aquac. Nutr.* **2009**, *15* (1), 94–103.

(81) Ryckebosch, E.; Bruneel, C.; Termote-Verhalle, R.; Lemahieu, C.; Muylaert, K.; Van Durme, J.; Goiris, K.; Foubert, I. Stability of Omega-3 LC-PUFA-Rich Photoautotrophic Microalgal Oils Compared to Commercially Available Omega-3 LC-PUFA Oils. *J. Agric. Food Chem.* **2013**, *61* (42), 10145–10155.

(82) Cho, S. Y.; Joo, D. S.; Choi, H. G.; Naraand, E.; Miyashita, K. Oxidative Stability of Lipids from Squid Tissues. *Fish. Sci.* **2001**, *67* (4), 738–743.

(83) Lu, F. S. H.; Bruheim, I.; Jacobsen, C. New Parameters for Evaluating the Quality of Commercial Krill Oil Capsules from the Aspect of Lipid Oxidation and Non-Enzymatic Browning Reactions. *Eur. J. Lipid Sci. Technol.* **2015**, *117* (8), 1214–1224.

(84) Singh, J.; Saxena, R. C. An Introduction to Microalgae: Diversity and Significance. In *Handbook of Marine Microalgae: Biotechnology Advances*; Kim, S.-K., Ed.; Academic Press, **2015**; pp 11–24.

(85) Buono, S.; Langellotti, A. L.; Martello, A.; Rinna, F.; Fogliano, V. Functional Ingredients from Microalgae. *Food Funct.* **2014**, *5* (8), 1669–1685.

(86) Harwood, J. L. Algae: Critical Sources of Very Long-Chain Polyunsaturated Fatty Acids. *Biomolecules* **2019**, *9* (11), 1–14.

(87) Boelen, P.; van Mastrigt, A.; van de Bovenkamp, H. H.; Heeres, H. J.; Buma, A. G. J. Growth Phase Significantly Decreases the DHA-to-EPA Ratio in Marine Microalgae. *Aquac. Int.* **2017**, *25* (2), 577–587.

(88) Pignolet, O.; Jubeau, S.; Vaca-Garcia, C.; Michaud, P. Highly Valuable Microalgae: Biochemical and Topological Aspects. *J. Ind. Microbiol. Biotechnol.* **2013**, *40* (8), 781–796.

(89) Scholz, M. J.; Weiss, T. L.; Jinkerson, R. E.; Jing, J.; Roth, R.; Goodenough, U.; Posewitz, M. C.; Gerken, H. G. Ultrastructure and Composition of the Nannochloropsis Gaditana Cell Wall. *Eukaryot. Cell* **2014**, *13* (11), 1450–1464.

(90) Zhu, C. J.; Lee, Y. K. Determination of Biomass Dry Weight of Marine Microalgae. *J. Appl. Phycol.* **1997**, *9* (2), 189–194.

(91) Goold, H.; Beisson, F.; Peltier, G.; Li-Beisson, Y. Microalgal Lipid Droplets: Composition, Diversity, Biogenesis and Functions. *Plant Cell Rep.* **2015**, *34* (4), 545–555.

(92) Ryckebosch, E.; Bruneel, C.; Termote-Verhalle, R.; Goiris, K.; Muylaert, K.; Foubert, I. Nutritional Evaluation of Microalgae Oils Rich in Omega-3 Long Chain Polyunsaturated Fatty Acids as an Alternative for Fish Oil. *Food Chem.* **2014**, *160*, 393–400.

(93) Suali, E.; Sarbatly, R. Conversion of Microalgae to Biofuel. *Renew. Sustain. Energy Rev.* **2012**, *16* (6), 4316–4342.

(94) Bumbak, F.; Cook, S.; Zachleder, V.; Hauser, S.; Kovar, K. Best Practices in Heterotrophic High-Cell-Density Microalgal Processes: Achievements, Potential and Possible Limitations. *Appl. Microbiol. Biotechnol.* **2011**, *91* (1), 31–46.

(95) Balduyck, L.; Bijttebier, S.; Bruneel, C.; Jacobs, G.; Voorspoels, S.; Van Durme, J.; Muylaert, K.; Foubert, I. Lipolysis in T-Isochrysis Lutea during Wet Storage at Different Temperatures. *Algal Res.* **2016**, *18*, 281–287.

(96) Sati, H.; Mitra, M.; Mishra, S.; Baredar, P. Microalgal Lipid Extraction Strategies for Biodiesel Production: A Review. *Algal Res.* **2019**, *38* (101413).

(97) de Carvalho, J. C.; Magalhães, A. I.; de Melo Pereira, G. V.; Medeiros, A. B. P.; Sydney, E. B.; Rodrigues, C.; Aulestia, D. T. M.; de Souza Vandenberghe, L. P.; Soccol, V. T.; Soccol, C. R. Microalgal Biomass Pretreatment for Integrated Processing into Biofuels, Food, and Feed. *Bioresour. Technol.* **2020**, *300* (122719).

(98) Shah, M. R.; Lutzu, G. A.; Alam, A.; Sarker, P.; Kabir Chowdhury, M. A.; Parsaeimehr, A.; Liang, Y.; Daroch, M. Microalgae in Aquafeeds for a Sustainable Aquaculture Industry. *J. Appl. Phycol.* **2017**, *30* (1), 197–213.

(99) Winwood, R. J. Recent Developments in the Commercial Production of DHA and EPA Rich Oils from Micro-Algae. *OCL - Oilseeds fats, Crop. lipids* **2013**, *20* (6).

(100) European Commission. Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 Establishing the Union List of Novel Foods in Accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on Novel Foods. *Off. J. Eur. Union* **2017**, *L351*, 72–201.

(101) Sidari, R.; Tofalo, R. A Comprehensive Overview on Microalgal-Fortified/Based Food and Beverages. *Food Rev. Int.* **2019**, *35* (8), 778–805.

(102) European Commission. *Summary of the applications submitted within the meaning of Article 10(1) of Regulation (EU) 2015/2283*.

(103) Zanella, L.; Vianello, F. Microalgae of the Genus Nannochloropsis: Chemical Composition and Functional Implications for Human Nutrition. *J. Funct. Foods* **2020**, *68*, 103919.

(104) Hemaiswarya, S.; Raja, R.; Kumar, R. R.; Ganesan, V.; Anbazhagan, C. Microalgae: A Sustainable Feed Source for Aquaculture. *World J. Microbiol. Biotechnol.* **2011**, *27* (8), 1737–1746.

(105) Muller-Feuga, A. Microalgae for Aquaculture: The Current Global Situation and Future Trends. In *Handbook of Microalgal Culture: Applied Phycology and Biotechnology: Second Edition*; Richmond, A., Hu, Q., Eds.; Blackwell, **2013**; pp 615–627.

(106) Liang, Y.; Sarkany, N.; Cui, Y. Biomass and Lipid Productivities of Chlorella Vulgaris under Autotrophic, Heterotrophic and Mixotrophic Growth Conditions. *Biotechnol. Lett.* **2009**, *31* (7), 1043–1049.

(107) Schade, S.; Stangl, G. I.; Meier, T. Distinct Microalgae Species for Food—Part 2: Comparative Life Cycle Assessment of Microalgae and Fish for Eicosapentaenoic Acid (EPA), Docosahexaenoic Acid (DHA), and Protein. *J. Appl. Phycol.* **2020**, *32* (5), 2997–3013.

(108) Ryckebosch, E.; Muylaert, K.; Foubert, I. Optimization of an Analytical Procedure for Extraction of Lipids from Microalgae. *JAOCS, J. Am. Oil Chem. Soc.* **2012**, *89* (2), 189–198.

(109) Bernaerts, T. M. M.; Verstreken, H.; Dejonghe, C.; Gheysen, L.; Foubert, I.; Grauwet, T.; Van Loey, A. M. Cell Disruption of Nannochloropsis Sp. Improves in Vitro Bioaccessibility of Carotenoids and Ω3-LC-PUFA. *J. Funct. Foods* **2020**, *65* (103770).

(110) Gheysen, L.; Bernaerts, T.; Bruneel, C.; Goiris, K.; Van Durme, J.; Van Loey, A.; De Cooman, L.; Foubert, I. Impact of Processing on N-3 LC-PUFA in Model Systems Enriched with Microalgae. *Food Chem.* **2018**, *2268*, 441–450.

(111) Glencross, B. D.; Baily, J.; Berntssen, M. H. G.; Hardy, R.; MacKenzie, S.; Tocher, D. R. Risk Assessment of the Use of Alternative Animal and Plant Raw Material Resources in Aquaculture Feeds. *Rev. Aquac.* **2020**, *12* (2), 703–758.

(112) Walker, C. G.; Jebb, S. A.; Calder, P. C. Stearidonic Acid as a Supplemental Source of ω-3 Polyunsaturated Fatty Acids to Enhance Status for Improved Human Health. *Nutrition* **2013**, *29* (2), 363–369.

(113) Ursin, V. M. Modification of Plant Lipids for Human Health: Development of Functional Land-Based Omega-3 Fatty Acids. *J. Nutr.* **2003**, *133* (12), 4271–4274.

(114) Kitessa, S. M.; Abeywardena, M.; Wijesundera, C.; Nichols, P. D. DHA-Containing Oilseed: A Timely Solution for the Sustainability Issues Surrounding Fish Oil Sources of the Health-Benefitting Long-Chain Omega-3 Oils. *Nutrients* **2014**, *6* (5), 2035–2058.

(115) Ruiz-Lopez, N.; Haslam, R. P.; Napier, J. A.; Sayanova, O. Successful High-Level Accumulation of Fish Oil Omega-3 Long-Chain Polyunsaturated Fatty Acids in a Transgenic Oilseed Crop. *Plant J.* **2014**, *77* (2), 198–208.

(116) Usher, S.; Han, L.; Haslam, R. P.; Michaelson, L. V.; Sturtevant, D.; Aziz, M.; Chapman, K. D.; Sayanova, O.; Napier, J. A. Tailoring Seed Oil Composition in the Real World: Optimising Omega-3 Long Chain Polyunsaturated Fatty Acid Accumulation in Transgenic Camelina Sativa. *Sci. Rep.* **2017**, *7* (1), 1–13.

(117) Petrie, J. R.; Zhou, X. R.; Leonforte, A.; McAllister, J.; Shrestha, P.; Kennedy, Y.; Belide, S.; Buzza, G.; Gororo, N.; Gao, W.; Lester, G.; Mansour, M. P.; Mulder, R. J.; Liu, Q.; Tian, L.; Silva, C.; Cogan, N. O. I.; Nichols, P. D.; Green, A. G.; de Feyter, R.; Devine, M. D.; Singh, S. P. Development of a Brassica Napus (Canola) Crop Containing Fish Oil-Like Levels of DHA in the Seed Oil. *Front. Plant Sci.* **2020**, *11* (June), 1–15.

(118) Xue, Z.; Sharpe, P. L.; Hong, S. P.; Yadav, N. S.; Xie, D.; Short, D. R.; Damude, H. G.; Rupert, R. A.; Seip, J. E.; Wang, J.; Pollak, D. W.; Bostick, M. W.; Bosak, M. D.; Macool, D. J.; Hollerbach, D. H.; Zhang, H.; Arcilla, D. M.; Bledsoe, S. A.; Croker, K.; McCord, E. F.; Tyreus, B. D.; Jackson, E. N.; Zhu, Q. Production of Omega-3 Eicosapentaenoic Acid by Metabolic Engineering of Yarrowia Lipolytica. *Nat. Biotechnol.* **2013**, *31* (8), 734–740.

(119) Tan, K.; Ma, H.; Li, S.; Zheng, H. Bivalves as Future Source of Sustainable Natural Omega-3 Polyunsaturated Fatty Acids. *Food Chem.* **2020**, *311* (125907).

(120) Spicer, A.; Molnar, A. Gene Editing of Microalgae: Scientific Progress and Regulatory Challenges in Europe. *Biology (Basel).* **2018**, *7* (1), 21.

(121) Petrie, J. R.; Shrestha, P.; Zhou, X. R.; Mansour, M. P.; Liu, Q.; Belide, S.; Nichols, P. D.; Singh, S. P. Metabolic Engineering Plant Seeds with Fish Oil-Like Levels of DHA. *PLoS One* **2012**, *7* (11), 2–8.

(122) Zhou, X. R.; Li, J.; Wan, X.; Hua, W.; Singh, S. Harnessing Biotechnology for the Development of New Seed Lipid Traits in Brassica. *Plant Cell Physiol.* **2019**, *60* (6), 1197–1204.

(123) Mitchell, M. C.; Pritchard, J.; Okada, S.; Zhang, J.; Venables, I.; Vanhercke, T.; Ral, J. P. Increasing Growth and Yield by Altering Carbon Metabolism in a Transgenic Leaf Oil Crop. *Plant Biotechnol. J.* **2020**, *2*, 1–11.

(124) Vanhercke, T.; Dyer, J. M.; Mullen, R. T.; Kilaru, A.; Rahman, M. M.; Petrie, J. R.; Green, A. G.; Yurchenko, O.; Singh, S. P. Metabolic Engineering for Enhanced Oil in Biomass. *Prog. Lipid Res.* **2019**, *74* (February), 103–129.

(125) Colombo, S. M.; Campbell, L. G.; Murphy, E. J.; Martin, S. L.; Arts, M. T. Potential for Novel Production of Omega-3 Long-Chain Fatty Acids by Genetically Engineered Oilseed Plants to Alter Terrestrial Ecosystem Dynamics. *Agric. Syst.* **2018**, *164* (March), 31–37.

(126) Betancor, M. B.; Li, K.; Bucerzan, V. S.; Sprague, M.; Sayanova, O.; Usher, S.; Han, L.; Norambuena, F.; Torrissen, O.; Napier, J. A.; Tocher, D. R.; Olsen, R. E. Oil from Transgenic Camelina Sativa Containing over 25 % N-3 Long-Chain PUFA as the Major Lipid Source in Feed for Atlantic Salmon (Salmo Salar). *Br. J. Nutr.* **2018**, *119* (12), 1378–1392.

(127) GOED. *Global Recommendations for EPA and DHA Intake*.

(128) Garg, M. L.; Wood, L. G.; Singh, H.; Moughan, P. J. Means of Delivering Recommended Levels of Long Chain N-3 Polyunsaturated Fatty Acids in Human Diets. *J. Food Sci.* **2006**, *71* (5).

(129) Salem, N.; Eggersdorfer, M. Is the World Supply of Omega-3 Fatty Acids Adequate for Optimal Human Nutrition? *Curr. Opin. Clin. Nutr. Metab. Care* **2015**, *18* (2), 147–154.

(130) Sioen, I.; Devroe, J.; Inghels, D.; Terwecoren, R.; De Henauw, S. The Influence of N-3 PUFA Supplements and n-3 PUFA Enriched Foods on the n-3 LC PUFA Intake of Flemish Women. *Lipids* **2010**, *45* (4), 313–320.

(131) Anbudhasan, P.; Surendraraj, A.; Karkuzhali, S.; Ramasamy, D. Development of Omega 3 Fatty Acid Enriched Stable Functional Foods: Challenges and Options. *Int. J. Innov. Res. Rev.* **2014**, *2* (1), 1–13.

(132) Fraeye, I.; Bruneel, C.; Lemahieu, C.; Buyse, J.; Muylaert, K.; Foubert, I. Dietary Enrichment of Eggs with Omega-3 Fatty Acids: A Review. *Food Res. Int.* **2012**, *48* (2), 961–969.

(133) Papadopoulos, G.; Goulas, C.; Apostolaki, E.; Abril, R. Effects of Dietary Supplements of Algae, Containing Polyunsaturated Fatty Acids, on Milk Yield and the Composition of Milk Products in Dairy Ewes. *J. Dairy Res.* **2002**, *69*, 357–365.

(134) Meale, S. J.; Chaves, A. V.; He, M. L.; McAllister, T. A. Dose-Response of Supplementing Marine Algae (Schizochytrium Spp.) on Production Performance, Fatty Acid Profiles, and Wool Parameters of Growing Lambs. *J. Anim. Sci.* **2014**, *92* (5), 2202–2213.

(135) Farhoosh, R. New Insights into the Kinetic and Thermodynamic Evaluations of Lipid Peroxidation. *Food Chem.* **2022**, *375* (131659).

(136) Shahidi, F.; Zhong, Y. Lipid Oxidation and Improving the Oxidative Stability. *Chem. Soc. Rev.* **2010**, *39* (11), 4067.

(137) Schaich, K. M. Thinking Outside the Classical Chain Reaction Box of Lipid Oxidation. **2012**, *24* (3), 55–58.

(138) Ghnimi, S.; Budilarto, E.; Kamal-Eldin, A. The New Paradigm for Lipid Oxidation and Insights to Microencapsulation of Omega-3 Fatty Acids. *Compr. Rev. Food Sci. Food Saf.* **2017**, *16* (6), 1206–1218.

(139) Matthäus, B. Oxidation of Edible Oils. In *Oxidation in Foods and Beverages and Antioxidant Applications*; Decker, E., Elias, R., McClements, J., Eds.; Woodhead Publishing, **2010**; pp 183–238.

(140) Choe, E.; Min, D. B. Chemistry and Reactions of Reactive Oxygen Species in Foods. *Crit. Rev. Food Sci. Nutr.* **2006**, *46* (1), 1–22.

(141) Jacobsen, C.; García-Moreno, P. J.; Yesiltas, B.; Sørensen, A.-D. M. Lipid Oxidation and Traditional Methods for Evaluation. In *Omega-3 Delivery Systems*; García-Moreno, P. J., Jacobsen, C., Sorensen, A.-D. M., Yesiltas, B., Eds.; Academic Press, **2021**; pp 183–200.

(142) Johnson, D. R.; Decker, E. A. The Role of Oxygen in Lipid Oxidation Reactions: A Review. *Annu. Rev. Food Sci. Technol.* **2015**, *6* (1), 171–190.

(143) Schaich, K. M.; Shahidi, F.; Zhong, Y.; Eskin, N. A. M. Lipid Oxidation. In *Biochemistry of Foods*; Eskin, N. A. M., Ed.; Elsevier, **2013**; pp 419–478.

(144) Cui, L.; Decker, E. A. Phospholipids in Foods: Prooxidants or Antioxidants? *J. Sci. Food Agric.* **2016**, *96* (1), 18–31.

(145) Hazahari, N. Y. B.; Hosokawa, M.; Miyashita, K. Comparison of Oxidative Stability of Monogalactosyl Diacylglycerol, Digalactosyl Diacylglycerol, and Triacylglycerol Containing Polyunsaturated Fatty Acids. *Food Nutr. Sci.* **2018**, *09* (03), 221–234.

(146) Wijesundera, C.; Ceccato, C.; Watkins, P.; Fagan, P.; Fraser, B.; Thienthong, N.; Perlmutter, P. Docosahexaenoic Acid Is More Stable to Oxidation When Located at the Sn-2 Position of Triacylglycerol Compared to Sn-1(3). *JAOCS, J. Am. Oil Chem. Soc.* **2008**, *85* (6), 543–548.

(147) Farhoosh, R.; Niazmand, R.; Rezaei, M.; Sarabi, M. Kinetic Parameter Determination of Vegetable Oil Oxidation under Rancimat Test Conditions. *Eur. J. Lipid Sci. Technol.* **2008**, *110* (6), 587–592.

(148) Sullivan, J. C.; Budge, S. M.; St-Onge, M. Modeling the Primary Oxidation in Commercial Fish Oil Preparations. *Lipids* **2011**, *46* (1), 87–93.

(149) Jodko-Piórecka, K.; Cedrowski, J.; Litwinienko, G. Physico-Chemical Principles of Antioxidant Action, Including Solvent and Matrix Dependence and Interfacial Phenomena. In *Measurement of Antioxidant Activity & Capacity*; Apak, R., Capanoglu, E., Shahidi, F., Eds.; John Wiley & Sons, Ltd: Chichester, UK, **2017**; pp 225–272.

(150) Berton-carabin, C. C. Lipid Oxidation in Oil-in-Water Emulsions : Involvement of the Interfacial Layer. **2014**, *13*, 945–977.

(151) Dobarganes, M. C.; Velasco, J. Analysis of Lipid Hydroperoxides. *Eur. J. Lipid Sci. Technol.* **2002**, *104* (7), 420–428.

(152) Kamal Eldin, A. Methods to Determine the Extent of Lipid Oxidation in Foods. In *Oxidation in Foods and Beverages and Antioxidant Applications*; Decker, E. A., Ed.; Woodhead Publishing, **2010**; pp 181–195.

(153) Barriuso, B.; Astiasarán, I.; Ansorena, D. A Review of Analytical Methods Measuring Lipid Oxidation Status in Foods: A Challenging Task. *Eur. Food Res. Technol.* **2013**, *236* (1), 1–15.

(154) Abeyrathne, E. D. N. S.; Nam, K.; Ahn, D. U. Analytical Methods for Lipid Oxidation and Antioxidant Capacity in Food Systems. *Antioxidants* **2021**, *10* (10), 1–19.

(155) Symoniuk, E.; Ksibi, N.; Wroniak, M.; Lefek, M.; Ratusz, K. Oxidative Stability Analysis of Selected Oils from Unconventional Raw Materials Using Rancimat Apparatus. *Appl. Sci.* **2022**, *12* (10355).

(156) Gheysen, L.; Dejonghe, C.; Bernaerts, T.; Van Loey, A.; De Cooman, L.; Foubert, I. Measuring Primary Lipid Oxidation in Food Products Enriched with Colored Microalgae. *Food Anal. Methods* **2019**, *12* (10), 2150–2160.

(157) Karahadian, C.; Lindsay, R. C. Evaluation of Compounds Contributing Characterizing Fishy Flavors in Fish Oils. **1989**, *66* (7), 953–960.

(158) Ritter, J. C. S.; Budge, S. M. Key Lipid Oxidation Products Can Be Used to Predict Sensory Quality of Fish Oils with Different Levels of EPA and DHA. **2012**, *3*, 1169–1179.

(159) Phung, A. S.; Bannenberg, G.; Vigor, C.; Reversat, G.; Oger, C.; Roumain, M.; Galano, J.; Durand, T.; Muccioli, G. G.; Ismail, A.; Wang, S. C. Chemical Compositional Changes in Over-Oxidized Fish Oils. *Foods* **2020**, *9* (10), 1501.

(160) Lee. Analysis of Headspace Volatile and Oxidized Volatile Compounds in DHA-Enriched Fish Oil on Accelerated Oxidative Storage Introduction. **2003**, *68* (7), 2169–2177.

(161) Frankel, E. N. Volatile Lipid Oxidation Products. *Prog. Lipid Res.* **1982**, *22*, 1–33.

(162) Noble, A. C.; Nawar, W. W. Identification of Decomposition Products from Autoxidation of Methyl 4,7,10,13,16,19-Docosahexaenoate. *J. Am. Oil Chem. Soc.* **1975**, *52* (3), 92–95.

(163) Noble, A. C.; Nawar, W. W. The Autoxidation of Highly Unsaturated Fatty Acids: Methyl 4,7,10,13,16,19-Docosahexaenoate. *J. Am. Oil Chem. Soc.* **1971**, *48*, 800–803.

(164) Hsieh, R. J.; Kinsella, J. E. Oxidation of Polyunsaturated Fatty Acids: Mechanisms, Products, and Inhibition with Emphasis on Fish. *Adv. Food Nutr. Res.* **1989**, *33*, 233–341.

(165) Song, N. E.; Lee, J. Y.; Lee, Y. Y.; Park, J. D.; Jang, H. W. Comparison of Headspace–SPME and SPME-Arrow–GC–MS Methods for the Determination of Volatile Compounds in Korean Salt–Fermented Fish Sauce. *Appl. Biol. Chem.* **2019**, *62* (1).

(166) Marsili, R. T. Comparison of Solid-Phase Microextraction and Dynamic Headspace Methods for the Gas Chromatographic-Mass Spectrometric Analysis of Light-Induced Lipid Oxidation Products in Milk. *J. Chromatogr. Sci.* **1999**, *37* (1), 17–23.

(167) Kremser, A.; Jochmann, M. A.; Schmidt, T. C. Systematic Comparison of Static and Dynamic Headspace Sampling Techniques for Gas Chromatography. *Anal. Bioanal. Chem.* **2016**, *408* (24), 6567–6579.

(168) Lee, J. Y.; Kim, W. S.; Lee, Y. Y.; Choi, Y. S.; Choi, H.; Jang, H. W. Solid-Phase Microextraction Arrow for the Volatile Organic Compounds in Soy Sauce. *J. Sep. Sci.* **2019**, *42* (18), 2942–2948.

(169) Nam, T. G.; Lee, J. Y.; Kim, B. K.; Song, N. E.; Jang, H. W. Analyzing Volatiles in Brown Rice Vinegar by Headspace Solid-Phase Microextraction (SPME)–Arrow: Optimizing the Extraction Conditions and Comparisons with Conventional SPME. *Int. J. Food Prop.* **2019**, *22* (1), 1195–1204.

(170) Herrington, J. S.; Gómez-Ríos, G. A.; Myers, C.; Stidsen, G.; Bell, D. S. Hunting Molecules in Complex Matrices with Spme Arrows: A Review. *Separations* **2020**, *7* (1).

(171) Manousi, N.; Rosenberg, E.; Zachariadis, G. A. Solid-Phase Microextraction Arrow for the Sampling of Volatile Organic Compounds in Milk Samples. *Separations* **2020**, *7* (4), 1–12.

(172) Carocho, M.; Morales, P.; Ferreira, I. C. F. R. Antioxidants: Reviewing the Chemistry, Food Applications, Legislation and Role as Preservatives. *Trends Food Sci. Technol.* **2018**, *71* (October 2017), 107–120.

(173) Pérez-gálvez, A.; Viera, I.; Roca, M. Carotenoids and Chlorophylls as Antioxidants. *Antioxidants* **2020**, *9* (6), 1–39.

(174) Choe, E.; Min, D. B. Mechanisms of Antioxidants in the Oxidation of Foods. *Compr. Rev. Food Sci. Food Saf.* **2009**, *8* (4), 345–358.

(175) Stahl, W.; Sies, H. Antioxidant Activity of Carotenoids. *Mol. Aspects Med.* **2003**, *24* (6), 345–351.

(176) Miller, N. J.; Sampson, J.; Candeias, L. P.; Bramley, P. M.; Rice-Evans, C. A. Antioxidant Activities of Carotenes and Xanthophylls. *FEBS Lett.* **1996**, *384* (3), 240–242.

(177) Coulombier, N.; Jauffrais, T.; Lebouvier, N. Antioxidant Compounds from Microalgae: A Review. *Mar. Drugs* **2021**, *19* (10).

(178) Burton, G.; Ingold, K. Beta-Carotene: An Unusual Type of Lipid Antioxidant. *Science (80-.).* **1984**, *224* (4649), 569–573.

(179) Laguerre, M.; Bayrasy, C.; Panya, A.; Weiss, J.; McClements, D. J.; Lecomte, J.; Decker, E. A.; Villeneuve, P. What Makes Good Antioxidants in Lipid-Based Systems? The Next Theories Beyond the Polar Paradox. *Crit. Rev. Food Sci. Nutr.* **2015**, *55* (2), 183–201.

(180) Capanoglu, E.; Kamiloglu, S.; Ozkan, G.; Apak, R. Evaluation of Antioxidant Activity/ Capacity Measurement Methods for Food Products. In *Measurement of Antioxidant Activity and Capacity: Recent Trends and Applications*; Apak, R., Capanoglu, E., Shahidi, F., Eds.; John Wiley & Sons: Oxford, **2018**; pp 273–286.

(181) Gulcin, İ. Antioxidants and Antioxidant Methods: An Updated Overview. *Arch. Toxicol.* **2020**, *94* (3), 651–715.

(182) Goiris, K.; Muylaert, K.; De Cooman, L. Microalgae as a Novel Source of Antioxidants for Nutritional Applications. In *Handbook of Marine Microalgae*; Kim, S.-K., Ed.; Academic Press, **2015**; pp 269–280.

(183) Huang, J. J.; Lin, S.; Xu, W.; Cheung, P. C. K. Occurrence and Biosynthesis of Carotenoids in Phytoplankton. *Biotechnol. Adv.* **2017**, *35* (5), 597–618.

(184) Varela, J. C.; Pereira, H.; Vila, M.; León, R. Production of Carotenoids by Microalgae: Achievements and Challenges. *Photosynth. Res.* **2015**, *125* (3), 423–436.

(185) Liebler, D. C. Antioxidant Reactions of Carotenoids. *Ann. N. Y. Acad. Sci.* **1993**, *691* (1), 20–31.

(186) Liao, F.-X.; Hu, C.-H. A Density Functional Theory Study for the Role of End Groups on the Antioxidative Potency of Carotenoids. *Theor. Chem. Acc.* **2013**, *132* (5), 1–13.

(187) Nakagawa, K.; Fujimoto, K.; Miyazawa, T. β-Carotene as a High-Potency Antioxidant to Prevent the Formation of Phospholipid Hydroperoxides in Red Blood Cells of Mice. *Biochim. Biophys. Acta - Lipids Lipid Metab.* **1996**, *1299* (1), 110–116.

(188) Woodall, A. A.; Lee, S. W. M.; Weesie, R. J.; Jackson, M. J.; Britton, G. Oxidation of Carotenoids by Free Radicals: Relationship between Structure and Reactivity. *Biochim. Biophys. Acta - Gen. Subj.* **1997**, *1336* (1), 33–42.

(189) Mortensen, A.; Skibsted, L. H. Importance of Carotenoid Structure in Radical-Scavenging Reactions. *J. Agric. Food Chem.* **1997**, *45* (8), 2970–2977.

(190) Müller, L.; Fröhlich, K.; Böhm, V. Comparative Antioxidant Activities of Carotenoids Measured by Ferric Reducing Antioxidant Power (FRAP), ABTS Bleaching Assay (ATEAC), DPPH Assay and Peroxyl Radical Scavenging Assay. *Food Chem.* **2011**, *129* (1), 139–148.

(191) Goiris, K.; Muylaert, K.; Voorspoels, S.; Noten, B.; De Paepe, D.; E Baart, G. J.; De Cooman, L. Detection of Flavonoids in Microalgae from Different Evolutionary Lineages. *J. Phycol.* **2014**, *50* (3), 483–492.

(192) Shahidi, F.; Ambigaipalan, P. Phenolics and Polyphenolics in Foods, Beverages and Spices: Antioxidant Activity and Health Effects – A Review. *J. Funct. Foods* **2015**, *18*, 820–897.

(193) GOED. *Global Organization for EPA & DHA omega-3s, Best-Practice Guidelines on Oxidation Control*.

(194) Drusch, S.; Groß, N.; Schwarz, K. Efficient Stabilization of Bulk Fish Oil Rich in Long-Chain Polyunsaturated Fatty Acids. *Eur. J. Lipid Sci. Technol.* **2008**, *110* (4), 351–359.

(195) Gheysen, L.; Durnez, N.; Devaere, J.; Bernaerts, T.; Van Loey, A.; De Cooman, L.; Foubert, I. Oxidative Stability of Vegetable Purees Enriched with N-3-LC-PUFA Microalgal Biomass: Impact of Type of Vegetable. *Int. J. Food Sci. Technol.* **2019**.

(196) Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant Potential of Microalgae in Relation to Their Phenolic and Carotenoid Content. *J. Appl. Phycol.* **2012**, *24* (6), 1477–1486.

(197) Safafar, H.; Wagenen, J. Van; Møller, P.; Jacobsen, C. Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater. *Mar. Drugs* **2015**, *13* (12), 7339–7356.

(198) Bernaerts, T. M. M.; Gheysen, L.; Foubert, I.; Hendrickx, M. E.; Van Loey, A. M. Evaluating Microalgal Cell Disruption upon Ultra High Pressure Homogenization. *Algal Res.* **2019**, *42* (101616).

(199) Tesson, B.; Genet, M. J.; Fernandez, V.; Degand, S.; Rouxhet, P. G.; Martin-Jézéquel, V. Surface Chemical Composition of Diatoms. *ChemBioChem* **2009**, *10* (12), 2011–2024.

(200) Gheysen, L.; Lagae, N.; Devaere, J.; Goiris, K.; Goos, P.; Bernaerts, T.; Van Loey, A.; De Cooman, L.; Foubert, I. Impact of Nannochloropsis Sp. Dosage Form on the Oxidative Stability of n-3 LC-PUFA Enriched Tomato Purees. *Food Chem.* **2019**, *279*, 389–400.

(201) Uluata, S.; McClements, D. J.; Decker, E. A. Physical Stability, Autoxidation, and Photosensitized Oxidation of ω-3 Oils in Nanoemulsions Prepared with Natural and Synthetic Surfactants. *J. Agric. Food Chem.* **2015**, *63* (42), 9333–9340.

(202) Uluata, S.; Durmaz, G.; Julian McClements, D.; Decker, E. A. Comparing DPPP Fluorescence and UV Based Methods to Assess Oxidation Degree of Krill Oil-in-Water Emulsions. *Food Chem.* **2021**, *339* (127898).

(203) Uluata, S.; Mcclements, D. J.; Decker, E. A. Riboflavin-Induced Oxidation in Fish Oil-in-Water Emulsions : Impact of Particle Size and Optical Transparency. *Food Chem.* **2016**, *213*, 457–461.

(204) Lee, S.; Min, D. B. Effects, Quenching Mechanisms, and Kinetics of Carotenoids in Chlorophyll-Sensitized Photooxidation of Soybean Oil. *J. Agric. Food Chem.* **1990**, *38*, 1630–1634.

(205) Oh, S.; Lee, E.; Choe, E. Light Effects on Lipid Oxidation, Antioxidants, and Pigments in Dried Laver (Porphyra) during Storage. *Food Sci. Biotechnol.* **2014**, *23* (3), 701–709.

(206) Bradley, D. G.; Kim, H. J.; Min, D. B. Effects, Quenching Mechanisms, and Kinetics of Water Soluble Compounds in Riboflavin Photosensitized Oxidation of Milk. *J. Agric. Food Chem.* **2006**, *54*, 6016–6020.

(207) Sang, W.; Jin, Z. T. Lipid Oxidation of Fish Liver Oil as Affected by Light, Antioxidants and Temperature. *J. Food Process. Preserv.* **2004**, *28* (1), 1–10.

(208) Sugawara, T.; Komura, Y.; Hagino, H.; Hirata, T. Phycoerythrin Contributes to the Photooxidation of Eicosapentaenoic Acid in Porphyra Yezoensis During Light Exposure. *J. Food Sci.* **2006**, *71* (6), 486–491.

(209) Boon, C. S.; McClements, D. J.; Weiss, J.; Decker, E. A. Factors Influencing the Chemical Stability of Carotenoids in Foods. *Crit. Rev. Food Sci. Nutr.* **2010**, *50* (6), 515–532.

(210) Gentile, M. P.; Blanch, H. W. Physiology and Xanthophyll Cycle Activity of Nannochloropsis Gaditana. *Biotechnol. Bioeng.* **2001**, *75* (1), 1–12.

(211) Shene, C.; Chisti, Y.; Vergara, D.; Burgos-Díaz, C.; Rubilar, M.; Bustamante, M. Production of Eicosapentaenoic Acid by Nannochloropsis Oculata: Effects of Carbon Dioxide and Glycerol. *J. Biotechnol.* **2016**, *239*, 47–56.

(212) Kuczynska, P.; Jemiola-Rzeminska, M. Isolation and Purification of All-Trans Diadinoxanthin and All-Trans Diatoxanthin from Diatom Phaeodactylum Tricornutum. *J. Appl. Phycol.* **2017**, *29* (1), 79–87.

(213) European Commission. Commission Regulation (EU) Nr. 116/2010. *Off. J. Eur. Union* **2010**, *L37*, 16–18.

(214) Lee, H.; Kizito, S. A.; Weese, S. J.; Craig-Schmidt, M. C.; Lee, Y.; Wei, C. I.; An, H. Analysis of Headspace Volatile and Oxidized Volatile Compounds in DHA-Enriched Fish Oil on Accelerated Oxidative Storage. *J. Food Sci.* **2003**, *68* (7), 2169–2177.

(215) Cheung, L. K. Y.; Tomita, H.; Takemori, T. Mechanisms of Docosahexaenoic and Eicosapentaenoic Acid Loss from Pacific Saury and Comparison of Their Retention Rates after Various Cooking Methods. *J. Food Sci.* **2016**, *81* (8), C1899–C1907.

(216) Nawar, W. W. Thermal Degradation of Lipids. *J. Agric. Food Chem.* **1969**, *17* (1), 18–21.

(217) Fournier, V.; Destaillats, F.; Juanéda, P.; Dionisi, F.; Lambelet, P.; Sébédio, J.; Berdeaux, O. Thermal Degradation of Long-chain Polyunsaturated Fatty Acids during Deodorization of Fish Oil. *Eur. J. Lipid Sci. Technol.* **2006**, *108* (1), 33–42.

(218) Safafar, H.; Langvad, S.; Møller, P.; Jacobsen, C. Storage Conditions Affect Oxidative Stability and Nutritional Composition of Freeze-Dried Nannochloropsis Salina. *Eur. J. Lipid Sci. Technol.* **2017**, *119* (12).

(219) Lu, F. S. H.; Nielsen, N. S.; Baron, C. P.; Jacobsen, C. Marine Phospholipids: The Current Understanding of Their Oxidation Mechanisms and Potential Uses for Food Fortification. *Crit. Rev. Food Sci. Nutr.* **2017**, *57* (10), 2057–2070.

(220) Thomsen, B. R.; Haugsgjerd, B. O.; Griinari, M.; Lu, H. F. S.; Bruheim, I.; Vogt, G.; Oterhals, Å.; Jacobsen, C. Investigation of Oxidative Degradation and Non-Enzymatic Browning Reactions in Krill and Fish Oils. *Eur. J. Lipid Sci. Technol.* **2013**, *115* (12), 1357–1366.

(221) Cutignano, A.; Luongo, E.; Nuzzo, G.; Pagano, D.; Manzo, E.; Sardo, A.; Fontana, A. Profiling of Complex Lipids in Marine Microalgae by UHPLC/Tandem Mass Spectrometry. *Algal Res.* **2016**, *17*, 348–358.

(222) Belitz, H.-D.; Grosch, W.; Schieberle, P. Aroma Compounds. In *Food Chemistry*; Belitz, H. D., Grosch, W., Schieberle, P., Eds.; Springer: Heidelberg, **2009**; pp 340–402.

(223) Shahidi, F.; Zhong, Y. Lipid Oxidation: Measurement Methods. In *Bailey's Industrial Oil and Fat Products*; Shahidi, F., Ed.; Wiley: New Jersey, **2005**.

(224) Shahidi, F.; Zhong, Y. Revisiting the Polar Paradox Theory: A Critical Overview. *J. Agric. Food Chem.* **2011**, *59* (8), 3499–3504.

(225) Vejdan, A.; Ojagh, S. M.; Abdollahi, M. Effect of Gelatin/Agar Bilayer Film Incorporated with TiO2 Nanoparticles as a UV Absorbent on Fish Oil Photooxidation. *Int. J. Food Sci. Technol.* **2017**, *52* (8), 1862–1868.

(226) Rontani, J.; Belt, S. T. Photo- and Autoxidation of Unsaturated Algal Lipids in the Marine Environment: An Overview of Processes, Their Potential Tracers, and Limitations. *Org. Geochem.* **2020**, *139* (103941).

(227) Hsieh, T. C. Y.; Williams, S. S.; Vejaphan, W.; Meyers, S. P. Characterization of Volatile Components of Menhaden Fish (Brevoortia Tyrannus) Oil. **1989**, *66* (1), 114–117.

(228) Matthews, R. F.; Scanlan, R. A.; Libbey, L. M. Autoxidation Products of 2,4-Decadienal. *J. Am. Oil Chem. Soc.* **1971**, *48* (11), 745–747.

(229) Nogueira, M. S.; Scolaro, B.; Milne, G. L.; Castro, I. A. Oxidation Products from Omega-3 and Omega-6 Fatty Acids during a Simulated Shelf Life of Edible Oils. *LWT - Food Sci. Technol.* **2019**, *101*, 113–122.

(230) Lee, J. Y.; Min, S.; Choe, E. O.; Min, D. B. Formation of Volatile Compounds in Soy Flour by Singlet Oxygen Oxidation during Storage under Light. *J. Food Sci.* **2003**, *68* (6), 1933–1937.

(231) Rahmani, M.; Csallany, A. S. Role of Minor Constituents in the Photooxidation of Virgin Olive Oil. *J. Am. Oil Chem. Soc.* **1998**, *75* (7), 837–843.

(232) Lee, J.; Decker, E. A. Effects of Metal Chelator, Sodium Azide, and Superoxide Dismutase on the Oxidative Stability in Riboflavin-Photosensitized Oil-in-Water Emulsion Systems. *J. Agric. Food Chem.* **2011**, *59* (11), 6271–6276.

(233) Dodoo, D.; Tulashie, S. K.; Dodoo, T.; Kwaw, F. Assessing the Effects of Sunlight on the Photooxidation of Tropical Oils with Experimental and Computational Approaches. *JAOCS, J. Am. Oil Chem. Soc.* **2021**, *98* (4), 439–453.

(234) Harrysson, H.; Krook, J. L.; Larsson, K.; Tullberg, C.; Oerbekke, A.; Toth, G.; Pavia, H.; Undeland, I. Effect of Storage Conditions on Lipid Oxidation, Nutrient Loss and Colour of Dried Seaweeds, Porphyra Umbilicalis and Ulva Fenestrata, Subjected to Different Pretreatments. *Algal Res.* **2021**, *56* (May), 102295.

(235) Lennersten, M.; Lingnert, H. Influence of Wavelength and Packaging Material on Lipid Oxidation and Colour Changes in Low-Fat Mayonnaise. *LWT - Food Sci. Technol.* **2000**, *33* (4), 253–260.

(236) Martin, H. D.; Ruck, C.; Schmidt, M.; Sell, S.; Beutner, S.; Mayer, B.; Walsh, R. Chemistry of Carotenoid Oxidation and Free Radical Reactions. *Pure Appl. Chem.* **1999**, *71* (12), 2253–2262.

(237) Van Dyck, S. The Impact of Singlet Oxygen on Lipid Oxidation in Foods. In *Oxidation in foods and beverages and antioxidant applications - Volume 1: Understanding mechanisms of oxidation and antioxidant activity*; Decker, E. A., Elias, R. J., McClements, D. J., Eds.; Woodhead Publishing: Cambridge, **2010**; pp 57–75.

(238) Shimidzu, N.; Goto, M.; Miki, W. Carotenoids as Singlet Oxygen Quenchers in Marine Organisms. *Fish. Sci.* **1996**, *62* (1), 134–137.

(239) Koca, N.; Karadeniz, F.; Burdurlu, H. S. Effect of PH on Chlorophyll Degradation and Colour Loss in Blanched Green Peas. *Food Chem.* **2007**, *100* (2), 609–615.

(240) Jeffrey, S.; Hallegraeff, G. Chlorophyllase Distribution in Ten Classes of Phytoplankton: A Problem for Chlorophyll Analysis. *Mar. Ecol. Prog. Ser.* **1987**, *35*, 293–304.

(241) Thron, M.; Eichner, K.; Ziegleder, G. The Influence of Light of Different Wavelengths on Chlorophyll-Containing Foods. *LWT - Food Sci. Technol.* **2001**, *34*, 542–548.

(242) Yan, X.; Li, X.; Zhou, C.; Fan, X. Prevention of Fish Oil Rancidity by Phlorotannins from Sargassum Kjellmanianum. *J. Appl. Phycol.* **1996**, *8* (3), 201–203.

(243) Athukorala, Y.; Lee, K.-W.; Shahidi, F.; Heu, M. S.; Kim, H.-T.; Lee, J.-S.; Jeon, Y.-J. Antioxidant Efficacy of Extracts of an Edible Red Alga (Grateloupia Filicina) in Linoleic Acid and Fish Oil. *J. Food Lipids* **2003**, *10*, 313–327.

(244) Kindleysides, S.; Quek, S. Y.; Miller, M. R. Inhibition of Fish Oil Oxidation and the Radical Scavenging Activity of New Zealand Seaweed Extracts. *Food Chem.* **2012**, *133* (4), 1624–1631.

(245) Chakraborty, K.; Joseph, D.; Joseph, D. Changes in the Quality of Refined Fish Oil in an Accelerated Storage Study. *J. Aquat. Food Prod. Technol.* **2016**, *25* (7), 1155–1170.

(246) Chakraborty, K.; Joseph, D. Effects of Antioxidative Substances from Seaweed on Quality of Refined Liver Oil of Leafscale Gulper Shark, Centrophorus Squamosus during an Accelerated Stability Study. *Food Res. Int.* **2018**, *103* (May 2017), 450–461.

(247) Chakraborty, K.; Joseph, D. Effect of Antioxidant Compounds from Seaweeds on Storage Stability of C20-22 Polyunsaturated Fatty Acid Concentrate Prepared from Dogfish Liver Oil. *Food Chem.* **2018**, *260* (March), 135–144.

(248) Golmakani, M.-T.; Moosavi-Nasab, M.; Keramat, M.; Mohammadi, M.-A. Arthrospira Platensis Extract as a Natural Antioxidant for Improving Oxidative Stability of Common Kilka (Clupeonella Cultriventris Caspia) Oil. *Turkish J. Fish. Aquat. Sci.* **2018**, *18*, 81–90.

(249) Lee, Y. L.; Chuang, Y. C.; Su, H. M.; Wu, F. S. Freeze-Dried Microalgae of Nannochloropsis Oculata Improve Soybean Oil's Oxidative Stability. *Appl. Microbiol. Biotechnol.* **2013**, *97* (22), 9675–9683.

(250) Alavi, N.; Golmakani, M. T. Improving Oxidative Stability of Virgin Olive Oil by Addition of Microalga Chlorella Vulgaris Biomass. *J. Food Sci. Technol.* **2017**, *54* (8), 2464–2473.

(251) Alavi, N.; Golmakani, M. T. Evaluation of Synergistic Effect of Chlorella Vulgaris and Citric Acid on Oxidative Stability of Virgin Olive Oil. *Food Sci. Biotechnol.* **2017**, *26* (4), 901–910.

(252) Alavi, N.; Golmakani, M. T. Antioxidant Properties of Whole-Cell Spirulina (Arthrospira Platensis) Powder Expressed in Olive Oil under Accelerated Storage Conditions. *J. Appl. Phycol.* **2017**, *29*, 2971–2978.

(253) Alavi, N.; Golmakani, M. T. Improving Oxidative Stability of Olive Oil: Incorporation of Spirulina and Evaluation of Its Synergism with Citric Acid. *Grasas y Aceites* **2017**, *68* (1), 1–11.

(254) Morsy, M. K.; Morsy, O. M.; Elbarbary, H. A.; Saad, M. A. Enhancing of Oxidative Stability and Quality Attributes of Olive Oil Using Spirulina (Arthrospira Platensis) Nanoparticles. *Lwt* **2019**, *101* (March 2018), 444–455.

(255) Wang, L.; Yang, B.; Yan, B.; Yao, X. Supercritical Fluid Extraction of Astaxanthin from Haematococcus Pluvialis and Its Antioxidant Potential in Sunflower Oil. *Innov. Food Sci. Emerg. Technol.* **2012**, *13*, 120–127.

(256) Rao, A. R.; Sarada, R.; Ravishankar, G. A. Stabilization of Astaxanthin in Edible Oils and Its Use as an Antioxidant. *J. Sci. Food Agric.* **2007**, *87*, 957–965.

(257) Limón, P.; Malheiro, R.; Casal, S.; Acién-Fernández, F. G.; Fernández-Sevilla, J. M.; Rodrigues, N.; Cruz, R.; Bermejo, R.; Pereira, J. A. Improvement of Stability and Carotenoids Fraction of Virgin Olive Oils by Addition of Microalgae Scenedesmus Almeriensis Extracts. *Food Chem.* **2015**, *175*, 203–211.

(258) Gouveia, L.; Nobre, B. P.; Marcelo, F. M.; Mrejen, S.; Cardoso, M. T.; Palavra, A. F.; Mendes, R. L. Functional Food Oil Coloured by Pigments Extracted from Microalgae with Supercritical CO2. *Food Chem.* **2007**, *101* (2), 717–723.

(259) Lima Araújo, K. G.; Domingues, J. R.; Sabaa Srur, A. U. O.; Da Silva, A. J. R. Production of Antioxidants by Anabaena PCC 7119 and Evaluation of Their Protecting Activity against Oxidation of Soybean Oil. *Food Biotechnol.* **2006**, *20* (1), 65–77.

(260) Foo, S. C.; Yusoff, F. M.; Ismail, M.; Basri, M.; Yau, S. K.; Khong, N. M. H.; Chan, K. W.; Ebrahimi, M. Antioxidant Capacities of Fucoxanthin-Producing Algae as Influenced by Their Carotenoid and Phenolic Contents. *J. Biotechnol.* **2017**, *241*, 175–183.

(261) Frankel, E. N.; Satué-Gracia, T.; Meyer, A. S.; German, J. B. Oxidative Stability of Fish and Algae Oils Containing Long-Chain Polyunsaturated Fatty Acids in Bulk and in Oil-in-Water Emulsions. *J. Agric. Food Chem.* **2002**, *50* (7), 2094–2099.

(262) Junqueira, H.; Schroder, A. P.; Thalmann, F.; Klymchenko, A.; Mély, Y.; Baptista, M. S.; Marques, C. M. Molecular Organization in Hydroperoxidized POPC Bilayers. *Biochim. Biophys. Acta - Biomembr.* **2021**, *1863* (10).

(263) Kim, S. M.; Jung, Y. J.; Kwon, O. N.; Cha, K. H.; Um, B. H.; Chung, D.; Pan, C. H. A Potential Commercial Source of Fucoxanthin Extracted from the Microalga Phaeodactylum Tricornutum. *Appl. Biochem. Biotechnol.* **2012**, *166* (7), 1843–1855.

(264) Prestegard, S. K.; Erga, S. R.; Steinrücken, P.; Mjøs, S. A.; Knutsen, G.; Rohloff, J. Specific Metabolites in a Phaeodactylum Tricornutum Strain Isolated from Western Norwegian Fjord Water. *Mar. Drugs* **2016**, *14* (1), 1–17.

(265) Pohnert, G.; Boland, W. The Oxylipin Chemistry of Attraction and Defense in Brown Algae and Diatoms. *Nat. Prod. Rep.* **2002**, *19* (1), 108–122.

(266) Lyberg, A. M.; Fasoli, E.; Adlercreutz, P. Monitoring the Oxidation of Docosahexaenoic Acid in Lipids. *Lipids* **2005**, *40* (9), 969–979.

(267) Yamaguchi, T.; Sugimura, R.; Shimajiri, J.; Suda, M.; Abe, M.; Hosokawa, M.; Miyashita, K. Oxidative Stability of Glyceroglycolipids Containing Polyunsaturated Fatty Acids. *J. Oleo Sci.* **2012**, *61* (9), 505–513.

(268) Nalder, T. D.; Miller, M. R.; Packer, M. A. Changes in Lipid Class Content and Composition of Isochrysis Sp. (T-Iso) Grown in Batch Culture. *Aquac. Int.* **2015**, *23* (5), 1293–1312.

(269) Balduyck, L.; Veryser, C.; Goiris, K.; Bruneel, C.; Muylaert, K.; Foubert, I. Optimization of a Nile Red Method for Rapid Lipid Determination in Autotrophic, Marine Microalgae Is Species Dependent. *J. Microbiol. Methods* **2015**, *118*, 152–158.

(270) Ryckebosch, E.; Bruneel, C.; Termote-Verhalle, R.; Muylaert, K.; Foubert, I. Influence of Extraction Solvent System on the Extractability of Lipid Components from the Biomass of Nannochloropsis Gaditana. *J. Appl. Phycol.* **2014**, *26* (3), 1501–1510.

(271) Demets, R.; Van Broekhoven, S.; Gheysen, L.; Van Loey, A.; Foubert, I. The Potential of Phaeodactylum as a Natural Source of Antioxidants for Fish Oil Stabilization. *Foods* **2022**, *11* (10), 1461.

(272) Roberty, S.; Furla, P.; Plumier, J. C. Differential Antioxidant Response between Two Symbiodinium Species from Contrasting Environments. *Plant Cell Environ.* **2016**, *39* (12), 2713–2724.

(273) Sampaio, K. A.; Ayala, J. V.; Silva, S. M.; Ceriani, R.; Verhé, R.; Meirelles, A. J. A. Thermal Degradation Kinetics of Carotenoids in Palm Oil. *JAOCS, J. Am. Oil Chem. Soc.* **2013**, *90* (2), 191–198.

(274) Zhao, D.; Kim, S. M.; Pan, C. H.; Chung, D. Effects of Heating, Aerial Exposure and Illumination on Stability of Fucoxanthin in Canola Oil. *Food Chem.* **2014**, *145*, 505–513.

(275) Gong, M.; Bassi, A. Carotenoids from Microalgae: A Review of Recent Developments. *Biotechnol. Adv.* **2016**, *34* (8), 1396–1412.

(276) EFSA. Scientific Opinion on the Re-evaluation of Butylated Hydroxytoluene BHT (E 321) as a Food Additive. *EFSA J.* **2012**, *10* (3), 2588–2631.

(277) EFSA. Scientific Opinion on the Re-Evaluation of Butylated Hydroxyanisole - BHA (E 320) as a Food Additive. *EFSA J.* **2011**, *9* (10), 2392.

(278) EFSA. Scientific Opinion on the Re-evaluation of Tocopherol-rich Extract (E 306), A-tocopherol (E 307), Γ-tocopherol (E 308) and Δ-tocopherol (E 309) as Food Additives. *EFSA J.* **2015**, *13* (9), 4247–4365.

(279) EFSA. Scientific Opinion on the Re-evaluation of Mixed Carotenes (E 160a (i)) and Beta-carotene (E 160a (Ii)) as a Food Additive. *EFSA J.* **2012**, *10* (3), 1–67.

(280) EFSA. Scientific Opinion on the Re-Evaluation of Lutein (E 161b) as a Food Additive. *EFSA J.* **2010**, *8* (7), 1678.

(281) Mendes, M.; Navalho, S.; Ferreira, A.; Paulino, C.; Figueiredo, D.; Silva, D.; Gao, F.; Gama, F.; Bombo, G.; Jacinto, R.; Aveiro, S.; Schulze, P.; Gonçalves, A. T.; Pereira, H.; Gouveia, L.; Patarra, R.; Abreu, M. H.; Silva, J.; Navalho, J.; Varela, J.; Speranza, L. Algae as Food in Europe: An Overview of Species Diversity and Their Application. *Foods* **2022**, *11* (1871).

(282) Zittelli, G. C.; Mangini, S.; Unamunzaga, C.; Verdelho, V.; Cadoret, J.-P. Algae as Food in Europe. *Eur. Algae Biomass Assoc.* **2021**.

(283) Van Durme, J.; Goiris, K.; De Winne, A.; De Cooman, L.; Muylaert, K. Evaluation of the Volatile Composition and Sensory Properties of Five Species of Microalgae. *J. Agric. Food Chem.* **2013**, *61* (46), 10881–10890.

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Education

2018 – present	Doctoral Program in Bioscience Engineering M²S, Food & Lipids, KU Leuven KULAK, Kortrijk, Belgium *Photoautotrophic microalgae as a novel, oxidatively stable n-3 LC-PUFA source and the associated importance of endogenous carotenoids* Supervisor: Prof. dr. ir. Imogen Foubert
2016 – 2018	Master of Bioscience Engineering KU Leuven, Belgium Food Science and Nutrition
2013 – 2016	Bachelor of Bioscience Engineering KU Leuven, Belgium

Work experience

Research experience

2018 – present	PhD researcher (FWO Fellow) Food & Lipids, KU Leuven KULAK, Kortrijk, Belgium Microalgae, Lipid oxidation, Antioxidants, Chromatography (GC, HPLC) Participation at international conferences with posters and lectures
2020	Winner poster pitch price KULAK Research Afternoon
2018	Laureate VBI Student Research Awards

Educational experience

2022	Practical lab sessions Bio-Organic Chemistry 1st Bachelor Bioscience Engineering
2018 – 2022	Practical courses Chemical Thermodynamics 2nd Bachelor Bioscience Engineering, Chemistry 3rd Bachelor Biochemistry
2018 – 2022	Supervision Project work (Bachelor's thesis) 2nd Bachelor Bioscience Engineering
2019 – 2021	Supervision Master's thesis Simon Van Broekhoven 2nd Master Bioscience Engineering
2019 – 2020	Supervision Master's thesis Lore Bonneux 2nd Master Bioscience Engineering
2018 – 2020	Practical courses Transport Phenomena 2nd Bachelor Bioscience Engineering, Biology 3rd Bachelor Chemistry
2018 – 2020	Practical lab sessions Analytical Chemistry 2nd Bachelor Bioscience Engineering, Chemistry

Skills

Languages	Dutch (native) English (fluent) French (proficient)
Software	Sigmaplot, JMP Chromeleon MS Office
Analytics	Lipid oxidation, Chromatography (GC, HPLC), Mass Spectrometry
Other	Academic writing, Presentation

LIST OF PUBLICATIONS

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

Demets, R., Gheysen, L., Van Loey, A., & Foubert, I. Oxidative stability differences of aqueous model systems enriched with photoautotrophic n-3 LC-PUFA rich microalgae: the antioxidative role of endogenous carotenoids. *Food Research International*, submitted.

Demets, R., Gheysen, L., Van Loey, A., & Foubert, I. (2023). Antioxidative capacity of microalgal carotenoids for stabilizing n-3 LC-PUFA rich oil: Initial quantity is key. *Food Chemistry*, 406 (135044).

Demets, R., Van Broekhoven, S., Gheysen, L., Van Loey, A., & Foubert, I. (2022). The Potential of *Phaeodactylum* as a Natural Source of Antioxidants for Fish Oil Stabilization. *Foods*, 11 (10), 1461.

Demets, R., Bonneux, L., Dejonghe, C., Gheysen, L., Van Loey, A., & Foubert, I. (2022). Photo-Oxidative Stability of Aqueous Model Systems Enriched with Omega-3 Long-Chain Polyunsaturated Fatty Acid-Rich Microalgae as Compared to Autoxidative Stability. *Journal of Agricultural and Food Chemistry*, 70 (18), 5691–5700.

Gheysen, L., **Demets, R.**, Devaere, J., Bernaerts, T., Goos, P., Van Loey, A., De Cooman, L., & Foubert, I. (2019) Impact of Microalgal Species on the Oxidative Stability of N-3 LC-PUFA Enriched Tomato Puree. *Algal Research*, 40 (101502).

PUBLICATION IN ACADEMIC BOOK, RECOGNIZED PUBLISHER

Demets, R., & Foubert, I. (2021). Traditional and novel sources of long-chain omega-3 fatty acids. In P. J. García-Moreno, C. Jacobsen, A.-D. M. Sorensen, & B. Yesiltas (Eds.), *Omega-3 Delivery Systems* (pp. 3–23). Academic Press.

ACTIVE CONTRIBUTIONS TO INTERNATIONAL CONFERENCES

Oral presentations

Demets, R., Gheysen, L., Van Loey, A., & Foubert, I. (2022). Antioxidative capacity of microalgal carotenoids for stabilizing n-3 LC-PUFA rich oil: initial quantity is key. *AlgaEurope*, 13-15 December 2022, Rome, Italy.

Demets, R., Van Broekhoven, S., Gheysen, L., Van Loey, A., & Foubert, I. (2022). The Potential of *Phaeodactylum* as a Natural Source of Antioxidants for Fish Oil Stabilization. *4th International Symposium on Lipid Oxidation and Antioxidants*, 23-25 May 2022, Vigo, Spain.

Demets, R., Bonneux, L., Dejonghe, C., Gheysen, L., Van Loey, A., & Foubert, I. (2021). Comparison between the Photo- and Auto-oxidative Stability of Aqueous Model Systems Enriched with Photoautotrophic and Heterotrophic n-3 LC-PUFA Rich Microalgae. *18th EuroFedLipid Congress and Expo*, 18-21 October 2021, online.

Gheysen, L., **Demets, R.**, Devaere, J., Bernaerts, T., Goos, P., Van Loey, A., De Cooman, L., & Foubert, I. (2021). Impact of microalgal species on the oxidative stability of n-3 LC-PUFA enriched tomato puree. *American Oil Chemists' Society (AOCS) Annual Meeting*, 10-14 May 2021, online.

Poster presentations

Gheysen, L., **Demets, R.**, Devaere, J., Bernaerts, T., Van Loey, A., De Cooman, L., & Foubert, I. (2020). Oxidative stability of vegetable purees enriched with omega-3 (n-3-LC-PUFA) rich microalgae. *World Congress on Oils and Fats (WCOF)*, 9-12 February 2020, Sydney, Australia.

Gheysen, L., **Demets, R.**, Devaere, J., Bernaerts, T., Goos, P., Van Loey, A., De Cooman, L., & Foubert, I. (2018). Impact of microalgal species on the oxidative stability of n-3 LC-PUFA enriched tomato puree. *AlgaEurope*, 4-6 December 2018, Amsterdam, The Netherlands.