Oxidative stability of omega-3 long chain poly-unsaturated fatty acids in vegetable purees enriched with microalgae

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SAMENVATTING

Het belang van omega-3 langketen poly-onverzadigde vetzuren (n-3 LC PUFA) wordt steeds vaker onder de aandacht gebracht aangezien ze bijdragen tot de hersenontwikkeling en de preventie van hart- en vaatziekten [1]. Deze gezondheidseffecten worden hoofdzakelijk toegewezen aan eicosapentaeenzuur (EPA, C20:5 n-3) en docosahexaeenzuur (DHA, C22:6 n-3), waarvan wordt aangeraden dagelijks 250 mg te consumeren [2]. Momenteel is vis de belangrijkste bron van n-3 LC-PUFA, maar deze staat de laatste tijd eerder in een negatief daglicht ten gevolge van overbevissing en de accumulatie van toxische componenten (PCB's, dioxines, lood, …) [3]. Microalgen zijn de primaire producenten van n-3 LC-PUFA en hebben reeds hun potentieel getoond als alternatieve, duurzame en vegetarische (en zelfs veganistische) bron [4].

Het onderzoek naar het aanrijken van voedingsproducten met n-3 LC-PUFA-rijke microalgen is zeer beperkt. Bovendien focussen de reeds uitgevoerde studies op een klein aantal verschillende voedingsproducten (hoofdzakelijk koolhydraat- en proteïnerijke producten), en werd de oxidatieve stabiliteit van deze producten niet onderzocht. Dit laatste is opmerkelijk aangezien n-3 LC-PUFA zeer gevoelig zijn aan lipidenoxidatie, een proces dat verhinderd moet worden. Het leidt immers tot een ongewenste geur en smaak, de aanwezigheid van toxische componenten en verlies van nutritionele waarde [5].

Het doel van dit doctoraatsonderzoek was om het potentieel van microalgen als alternatieve bron van n-3 LC-PUFA in groentengebaseerde producten in detail te onderzoeken. Deze voedingsproducten zijn essentieel in een gezond dieet en bevatten hoogwaardige componenten zoals vezels en antioxidanten [6]. Deze voedingsproducten aanrijken met n-3 LC-PUFA zou bijgevolg interessant zijn. Dit onderzoek heeft de nadruk gelegd op het bestuderen van de invloed van toedieningsvorm, microalgensoort en type groentenpuree op de n-3 LC-PUFA- en endogene antioxidantconcentratie. Daarnaast werd de oxidatieve stabiliteit van de

aangerijkte groentenpurees onderzocht. Om de primaire lipidenoxidatie in voedingsproducten aangerijkt met gekleurde fotoautotrofe microalgen op te volgen, werd een eenvoudige en betaalbare methode ontwikkeld in hoofdstuk 3.

In hoofdstuk 4 werden aangezuurde modelsystemen onderzocht, terwijl in hoofdstukken 5 – 7 reële groentenpurees werden bestudeerd. De belangrijkste conclusies van het onderzoek op de modelsystemen, waren ook van toepassing op de reële groentenpurees, met uitzondering van de daling van de hoeveelheid n-3 LC-PUFA door thermische degradatie in de modelsystemen.

In groentenpurees werd het gehalte aan n-3 LC-PUFA, carotenoïden, fenolische componenten en tocoferolen niet beïnvloed door verschillende procesintensiteiten, terwijl het gehalte aan ascorbinezuur drastisch daalde door een pasteurisatie of sterilisatie. Wanneer er *Nannochloropsis* olie als toedieningsvorm werd gebruikt, werden fysische verliezen door het blijven kleven aan het glaswerk, de mixer, de tubes van de hogedrukhomogenizator, etc. waargenomen. De fysische verliezen bij het gebruik van *Nannochloropsis* biomassa waren daarentegen opmerkelijk lager, waardoor deze als een meer gebruiksvriendelijke toedieningsvorm werd beschouwd.

Dit doctoraatsonderzoek bracht eveneens belangrijke inzichten bij omtrent de oxidatieve stabiliteit van groentenpurees aangerijkt met microalgen. De impact van verschillende procesintensiteiten op de oxidatieve stabiliteit van aangerijkte groentenpurees was zeer beperkt. De microalgensoort had daarentegen een cruciale impact op de oxidatieve stabiliteit van de groentenpuree.

Groentenpurees aangerijkt met fotoautotrofe microalgenbiomassa bleven oxidatief stabiel, onafhankelijk van het type groentenpuree en microalgensoort. Daarnaast vertoonde de toedieningsvorm een significante impact aangezien (partieel gedisrupteerde) biomassa een hogere oxidatieve stabiliteit vertoonde dan olie. De aanwezigheid van

carotenoïden (xanthofyllen, onafhankelijk van het type, en/of β-caroteen) uit de microalgen zelf, leidde tot een hogere oxidatieve stabiliteit van deze aangerijkte groentenpurees. Deze carotenoïden degradeerden reeds drastisch tijdens de eerste fase van het bewaarexperiment, wat hun functie als belangrijk antioxidant bevestigde. Daarnaast werd een statistisch model opgesteld dat de graad van oxidatie (afhankelijke variabele) uitdrukt aan de hand van verschillende potentieel beïnvloedende factoren (vrije vetzuren, carotenoïden, fenolische componenten, tocoferolen en ascorbinezuur) (*adjusted* R^2 van 82.3%). De variabelen die in dit model weerhouden werden, waren allemaal carotenoïden. Dit bevestigde opnieuw de hypothese dat de carotenoïden afkomstig van de microalgen cruciaal waren bij het behouden van de oxidatieve stabiliteit. Hun apolaire karakter kon hun belangrijke rol in het bewaren van de oxidatieve stabiliteit in een polaire omgeving partieel verklaren. Doorheen dit doctoraatsonderzoek werd een ondergeschikte rol van fenolische componenten, tocoferolen en ascorbinezuur in het behouden van de oxidatieve stabiliteit waargenomen.

Daarentegen was de antioxidantcapaciteit van de groentenpuree, ondanks de aanwezigheid van carotenoïden (bv. lycopeen), fenolische componenten en ascorbinezuur, onvoldoende om de n-3 LC-PUFA afkomstig van heterotrofe *Schizochytrium* biomassa of visolie, beiden zonder eigen carotenoïden, oxidatief stabiel te houden. Een vergelijking van de oxidatieve stabiliteit van groentenpuree en modelsystemen leerde ons dat de carotenoïden afkomstig uit de groentenpuree de oxidatieve stabiliteit hielpen te verhogen, maar dat deze onvoldoende waren voor het elimineren van oxidatie.

Finaal kon dus geconcludeerd worden dat fotoautotrofe microalgenbiomassa de meest geschikte bron is om groentenpurees rijk aan n-3 LC-PUFA en met een hoge oxidatieve stabiliteit te creëren. Op basis van de resultaten van het oxidatie-experiment en de Q_{10}-waarde werd voorspeld dat het product oxidatief stabiel zal blijven gedurende 2 jaar bij gekoelde bewaring (7°C) en gedurende 1 jaar bij bewaring op kamertemperatuur (17°C).

Een economische evaluatie van de met n-3 LC-PUFA aangerijkte groentenpurees toonde aan dat de bijkomende ingrediëntkost van de fotoautotrofe microalgen momenteel nog te hoog is. Verschillende onderzoekers voorspellen echter een daling van de productiekost van microalgen in de nabije toekomst [7,8]. Bovendien werden meerdere aanvragen ingediend om n-3 LC-PUFA-rijke microalgen goed te keuren onder de Europese *novel food* wetgeving [9]. Een reductie van de ingrediëntkost en goedkeuring door de huidige *novel food* wetgeving zal er toe leiden dat microalgen in competitie kunnen treden met de huidige n-3 LC-PUFA-bronnen. Bovendien bieden de fotoautotrofe microalgen het grote voordeel dat ze een hogere oxidatieve stabiliteit hebben dan de huidige commerciële bronnen. Fotoautotrofe microalgen zijn bijgevolg een veelbelovende alternatieve bron van n-3 LC-PUFA om de nutritionele waarde van onze voedingsproducten te verhogen.

ABSTRACT

Omega-3 long chain poly-unsaturated fatty acids (n-3 LC-PUFA) are getting more and more attention since their health benefits in the areas of brain development and in the prevention of cardiovascular disorders have been proven [1]. Mainly eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) are associated with these health benefits and a daily intake of 250 mg n-3 LC-PUFA is recommended [2]. Fish is the most well-known source of n-3 LC-PUFA, although it is under pressure due to a reducing stock and the accumulation of toxic compounds (PCB's, dioxins, lead,…) [3]. Microalgae, the primary producers of n-3 LC-PUFA, have already shown their potential as an alternative, sustainable, and even vegetarian and vegan, source [4].

Limited research has however been performed on food products enriched with n-3 LC-PUFA rich microalgae. This research moreover has only targeted a small range of food products (mainly carbohydrate and protein-rich food products), and did not study the oxidative stability of these food products. The latter is surprising given the high susceptibility of n-3 LC-PUFA to lipid oxidation, a process that should be avoided, since it results in an undesired flavor, the presence of toxic compounds, and a loss of nutritional value [5].

The aim of this PhD was to investigate in-depth the potential of microalgae as an alternative source of n-3 LC-PUFA in vegetable-based products. These products are essential in a healthy diet as they contain high value compounds like fibers and antioxidants [6]. The presence of the latter is especially interesting when enriching products with n-3 LC-PUFA. The focus was especially, on the impact of dosage form, microalgal species and type of vegetable puree on the n-3 LC-PUFA and endogenous antioxidant concentration and on the oxidative stability of the enriched vegetable purees. To monitor the primary lipid oxidation in food products enriched with colored, photoautotrophic microalgae, a simple and low-cost method was developed in chapter 3.

In chapter 4, model systems representing acidic products were investigated, while in chapters 5 – 7 real vegetable purees were used. The main conclusions drawn based on the model systems showed to be also valid in real purees, except for the decrease in the amount of n-3 LC-PUFA due to thermal degradation in the model systems.

In the vegetable purees, it was shown that the amount of n-3 LC-PUFA, carotenoids, phenolic compounds and tocopherols was not affected by processing, irrespective of the intensity, while the amount of ascorbic acid was drastically reduced by pasteurization or sterilization. Large physical losses by stickiness to glassware, mixer, tubes of the high pressure homogenizer, etc. were however observed when using *Nannochloropsis* oil as dosage form. In contrast, *Nannochloropsis* biomass showed lower physical losses and thus showed a higher usability.

This PhD research also uncovered some essential insights concerning the oxidative stability of vegetable purees enriched with microalgae. The impact of processing intensity was very limited, but a large difference was observed between purees enriched with photoautotrophic and heterotrophic microalgae.

Vegetable purees enriched with photoautotrophic microalgal biomass were oxidatively stable, irrespective of the type of vegetable puree and the microalgal species. A significant impact of dosage form was observed as (partially disrupted) biomass showed a higher oxidative stability compared to oil. The high oxidative stability of photoautotrophic microalgae, and in particular biomass, could primarily be attributed to the presence of carotenoids (xanthophylls, irrespective of type, and/or β-carotene) in the photoautotrophic biomass. Drastic degradations of these carotenoids in an early stage of storage confirmed their function as important antioxidant. Additionally, all variables retained in a statistical model linking the degree of lipid oxidation (dependent variable), and different potentially influencing variables (free fatty acids, carotenoids, phenolic compounds, tocopherols and ascorbic acid) (adjusted R^2 of 82.3%) were carotenoids, supporting the hypothesis that primarily the carotenoids derived from the photoautotrophic microalgae showed an

antioxidant capacity. Their non-polar character can partially explain their high antioxidant capacity in a polar environment. Throughout this PhD research, phenolic compounds, tocopherols and ascorbic acid seemed to have a minor role in the maintenance of the oxidative stability.

On the other hand, a vegetable puree, although rich in antioxidants like carotenoids (e.g. lycopene), phenolic compounds and ascorbic acid, was not able to keep n-3 LC-PUFA derived from heterotrophic *Schizochytrium* biomass or fish oil, lacking endogenous carotenoids, oxidatively stable. Carotenoids derived from the vegetable purees helped to improve the oxidative stability when compared to the model systems but this was still insufficient.

Taken all the results together, photoautotrophic microalgal biomass was the most appropriate source in order to create an n-3 LC-PUFA enriched vegetable puree with a high oxidative stability. The results of the oxidation experiment combined with the Q_{10} value predict an oxidatively stable product for at least 2 years when stored refrigerated (7°C) or 1 year when stored at room temperature (17°C).

Based on an economical evaluation of n-3 LC-PUFA enriched vegetable purees, it could be concluded that the supplemental ingredient cost of photoautotrophic microalgae is currently too high. However, different researchers predict a decrease in the production cost of microalgae in the near future [7,8]. Furthermore, several applications of n-3 LC-PUFA rich microalgae have been submitted to the European novel food regulation [9]. Reduction of the ingredient cost and the approval of n-3 LC-PUFA rich microalgae under the novel food regulation will make it possible to compete with the currently commercially available n-3 LC-PUFA sources. Next to this, photoautotrophic microalgae improve the oxidative stability compared to commercial sources. Photoautotrophic microalgae are thus a promising source of n-3 LC-PUFA in order to boost the nutritional value of our food products.

LIST OF ABBREVIATIONS

ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
AVOC	Algal volatile organic compounds
BHA	Butylated hydroxyanisol
BHT	Butylated hydroxytoluene
CD	Conjugated dienes
CT	Conjugated trienes
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester
FAO	Food agriculture organization of the United Nations
FID	Flame ionization detection
FOX	Ferrous oxidation xylenol orange
FRAP	Ferric reducing antioxidant power
GC	Gas chromatography
HPLC	High performance liquid chromatography
ICP-OES	Inductively coupled plasma - optical emission spectrometry
IDF	International Dairy Federation
LA	Linoleic acid
LPSC	Luminol-chemiluminescence based peroxyl radical scavenging assay

MS	Mass spectrometry
n-3 LC-PUFA	Omega-3 long chain poly-unsaturated fatty acids
n-6 LC-PUFA	Omega-6 long chain poly-unsaturated fatty acids
ORAC	Oxygen radical absorbance capacity
PAD	Photodiode array detection
SEC-RI	Size-exclusion chromatography - refractive index detection
SPME (HS-)	Solid phase micro-extraction (head space-)
SPE	Solid phase extraction
TBARS	Thiobarbituric acid reactive substances
TEAC	Trolox equivalent antioxidant capacity
TPP	Triphenylphosphine
WHO	World Health Organization

TABLE OF CONTENTS

CONTEXT & AIM

The health benefits of omega-3 long chain poly-unsaturated fatty acids (n-3 LC-PUFA) are generally accepted and are primarily associated with eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3). Their role in the prevention of cardiovascular diseases and in brain development has been proven in different epidemiological and clinical studies [1]. The Food and Agriculture Organization of the United Nations (FAO) recommends a daily intake of 250 mg n-3 LC-PUFA [2]. Food naturally rich in n-3 LC-PUFA, n-3 LC-PUFA enriched food and n-3 LC-PUFA supplements contribute to consuming a higher level of n-3 LC-PUFA. However, supplements only reach a small part of the population [10]. The consumption of foods naturally rich in n-3 LC-PUFA, fatty fish and fish oil, is low in most countries and a change in diet would be needed to meet the recommended intake [10,11]. In contrast, no change in eating habits is needed to enhance the intake of n-3 LC-PUFA via widely consumed enriched food products which reach a larger proportion of the population [12]. In this respect, there is an increased interest to enrich food products with n-3 LC-PUFA.

Microalgae, rich in n-3 LC-PUFA, could provide a sustainable alternative source to fish and fish oil, given reducing global fish stocks [4]. Microalgae are unicellular organisms, and the primary producers and a vegetarian source of n-3 LC-PUFA. They are characterized by a high growth rate and a limited competition with agriculture since they do not require arable land [13,14]. Microalgae can be cultivated in seawater or fresh water. Seawater microalgae do not depend on limited fresh water supplies and for microalgae that grow in fresh water, water demand is lower than for terrestrial crops because they do not actively transpire water [15]. Photoautotrophic microalgae obtain their energy from photosynthesis. During industrial production, they are cultivated under controlled circumstances in the presence of light and carbon dioxide. Besides n-3 LC-PUFA they contain other high value compounds such as antioxidants [4]. Heterotrophic microalgae grow by fermentation on

organic substrates without light [13]. Although they contain a high amount of lipids (70%), most of them lack carotenoids [16]. Each cultivation system has advantages and disadvantages, but in general, microalgae offer a great opportunity to innovate and create healthy food products enriched in n-3 LC-PUFA.

Due to their large number of double bounds, n-3 LC-PUFAs are, however, highly susceptible to lipid oxidation. Oxidation of n-3 LC-PUFA should be avoided, since it results in loss of nutritional value, undesired flavor and the presence of toxic compounds [5]. The presence of (natural) antioxidants in the food matrix can help to prevent the fatty acids from oxidation [17]. In this respect, fruit and vegetable-based products (for example soups, sauces, smoothies and juices) may be an interesting matrix given their relatively high antioxidant capacity. Moreover, fruit and vegetable-based products are essential in a healthy diet and also contain other high-value compounds, like micronutrients [6].

In this context, the objective of this PhD was to investigate the use of microalgae as an alternative source of n-3 LC-PUFA in order to boost the nutritional value of vegetable-based products. Part I comprises a literature review, while part II and III include experimental work. A schematic overview of this PhD is presented in Figure 1.

The literature study (**Part I**) is divided into two chapters. **Chapter 1** deals with the importance of n-3 LC-PUFA and their related health benefits. Additionally, the potential of microalgae as a source of n-3 LC-PUFA and some basic concepts about microalgae in general are described. Besides, this chapter gives an overview of the literature on the direct incorporation of n-3 LC-PUFA derived from microalgal species in food products. **Chapter 2** describes the main principles of lipid oxidation, its influencing factors and the most common measuring techniques.

In **Part II** (**chapter 3**) a simple and low-cost method to measure the primary lipid oxidation of food products enriched with colored photoautotrophic microalgae was developed. This method was then used throughout part III of the PhD.

The aim of **Part III** (chapters 4 – 7) was to acquire more insight in the effect of dosage form, microalgal species and type of vegetable puree on the amount of n-3 LC-PUFA and the endogenous antioxidants as well as on the oxidative stability of vegetable purees enriched with microalgae. Besides, the effect of processing typical for this type of food products was investigated.

Before studying the interaction of microalgae with vegetable purees, **chapter 4** investigated the potential different microalgal species in an aqueous acidic model system (representative of acidic products). This chapter studied the effect of mechanical (high pressure homogenization) followed by thermal (pasteurization) processing on n-3 LC-PUFA and the endogenous carotenoids. Additionally, the oxidative stability of the enriched suspensions was investigated.

The aim of chapters 5 – 7 was to study in-depth the potential of microalgae as an alternative source of n-3 LC-PUFA in real vegetable purees. These chapters investigated consecutively the impact of dosage form (**chapter 5**), the impact of microalgal species (**chapter 6**) and the impact of type of vegetable puree (**chapter 7**) on the amount of n-3 LC-PUFA and endogenous antioxidants, after different processing intensities, and the oxidative stability of the enriched vegetable purees.

Finally, in **chapter 8**, the main conclusions of the research conducted in this PhD and some future research possibilities have been summarized.

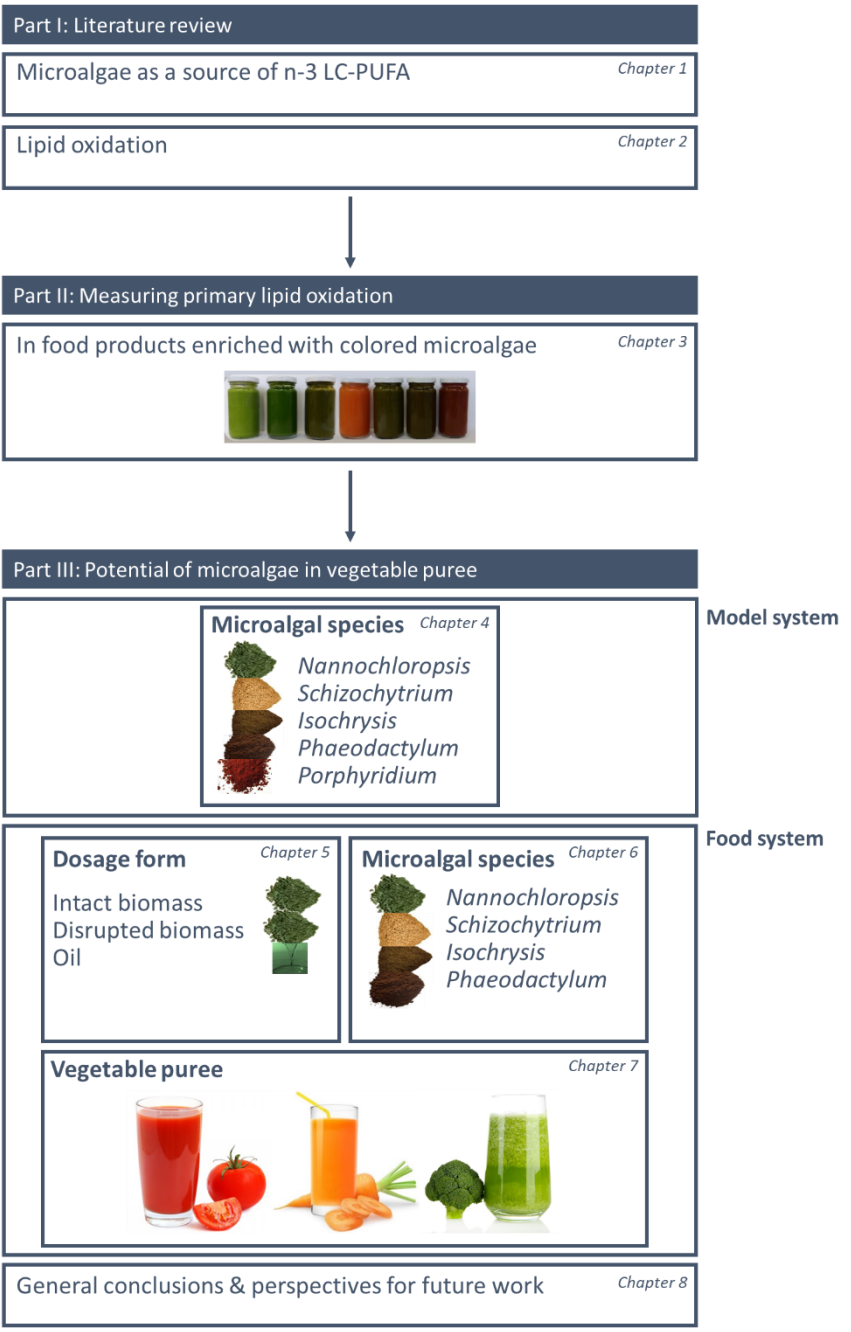


Figure 1. *Outline of this PhD*

PART I:

LITERATURE REVIEW

Chapter 1:

MICROALGAE AS A SOURCE OF OMEGA-3 LONG CHAIN POLY-UNSATURATED FATTY ACIDS

This chapter is based on:

Gheysen, L., Matton, V., Foubert, I. (2018). Chapter 1: Microalgae as a source of omega-3 poly-unsaturated fatty acids. In: Poly-unsaturated fatty acids (PUFAs): Food sources, health effects and significance in biochemistry (edited by A. Catala). Nova Science Publishers, USA.

1.1. HEALTH BENEFITS OF N-3 LC-PUFA

The health benefits of omega-3 poly-unsaturated fatty acids are generally accepted and are mainly associated with the consumption of omega-3 long chain poly-unsaturated fatty acids (n-3 LC-PUFA) containing a minimum of 20 carbon atoms, like eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) [1]. EPA and DHA respectively contain five and six double bounds, of which the first one is located on the third C-atom counting from the methyl end (Figure 1.1).

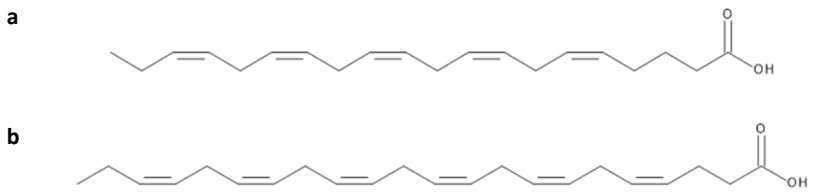


Figure 1.1. *The chemical structure of EPA (a) and DHA (b)*

Both fatty acids can be formed from the essential fatty acid alpha-linolenic acid (ALA, C18:3 n-3), which must be acquired through the diet, making EPA and DHA semi-essential fatty acids. A chain of desaturase and elongase enzymes in the human body is able to transform ALA into EPA and DHA [18]. This conversion is however very limited with conversion factors stated in literature varying between 0.01 and 10% [19]. The reason is that the pathway for the biosynthesis of n-3 LC-PUFA experiences competition from the linoleic acid (LA, C18:3 n-6) conversion to long chain omega-6 fatty acids (n-6 LC-PUFA) as both pathways are using the same enzymes for the biosynthesis of their corresponding long chain fatty acids [4]. The last decades the ratio n-6:n-3 in the western diet has become unbalanced with ratios typically 10:1 to 25:1 instead of the recommended 2:1 to 6:1 [20–22]. The direct consumption of EPA and DHA is indispensable, considering their low *in vivo* synthesis from ALA [11].

After consumption, EPA and DHA are mainly incorporated in the phospholipids of the cell membranes, where they influence the membrane fluidity. This leads to a change in membrane protein activity,

an impact on the hormone-receptor connection and the transport and signal regulation [1,4,18]. EPA and DHA are involved in the formation of precursors of anti-inflammatory immune responses and bioactive eicosanoids [1], while n-6 LC-PUFA lead to the formation of pro-inflammatory eicosanoids. A higher intake of n-3 LC-PUFA helps to restore the n-6:n-3 balance and therewith the suppression of pro-inflammatory compounds [23]. The European Food Safety Authority (EFSA) approved different health claims related to the consumption of EPA and DHA. A combined consumption of EPA and DHA contributes to normal functioning of the heart and cardiac related functions. Thereby, a normal blood pressure and a normal blood concentration of triacylglycerols is maintained. Consumption of only DHA contributes to the maintenance of normal vision and the development of a normal brain function. Maternal intake of DHA helps the development of the eye of the fetus and breastfed infants [24]. In literature, health benefits of EPA and DHA related to depression, dementia, rheumatoid arthritis, kidney functioning, etc. are described as well [19]. These benefits are however not yet approved by EFSA. The World Health Organization (WHO) and governments around the world have set a recommended daily intake of 250-500 mg of EPA and DHA based on the results on the prevention of cardiovascular disorders and cognitive development. Higher intakes are advised for pregnant and lactating woman [24–27]. Long-term intake of combined doses of EPA and DHA up to 5 g/day, and supplemental intakes of EPA alone up to 1.8 g/day, do not raise safety concerns for adults [24].

Food naturally rich in n-3 LC-PUFA, n-3 LC-PUFA enriched food and n-3 LC-PUFA supplements can attribute to consuming a higher level of n-3 LC-PUFA. Supplements only reach a small part of the population [10]. The consumption of foods naturally rich in n-3 LC-PUFA, fatty fish and fish oil, is low in most countries and a change in diet would be needed to meet the recommended intake [10,11]. In contrast, no change in eating habits is needed to enhance the intake of n-3 LC-PUFA via enriched food products, which reach a larger part of the population [12]. In this respect, there is an increased interest to enrich food products with a source of n-3 LC-PUFA.

Especially fatty fish, like herring and salmon are sources of EPA and DHA, with contents up to 1200 mg/100 g [18,28]. Also other marine sources, like krill and squid contain EPA and DHA [29]. However, fish, krill and squid do not produce n-3 LC-PUFA themselves, since they are lacking enzymes for the conversion of ALA to EPA and DHA. Besides, there is accumulation of PCB's, dioxins and lead in the marine food chain as well [3,30]. Moreover, the current fish stock is too low to provide the global population with the recommended daily intake. An alternative and sustainable source of n-3 LC-PUFA is thus needed. Microalgae are the primary producers of n-3 LC-PUFA, which accumulate along the food chain and can provide such an alternative [4,31,32].

1.2. MICROALGAE AS AN ALTERNATIVE SOURCE

Microalgae are unicellular aquatic organisms, growing in marine or fresh water. Most of them are eukaryotic and they exist individually, in chains or in groups [33]. Microalgae are featured by a large diversity, with an estimation of more than 200.000 species [34,35] differing amongst others in shape, size and cell wall composition [36].

Depending on the species, microalgae can be cultured photoautotrophically, heterotrophically and/or mixotrophically. The photoautotrophic growth of microalgae requires only light and CO_2 as an inorganic carbon source to produce organic material. Furthermore, some nutrients like nitrogen, sulphur and phosphorus are stimulating the growth. Via their photosynthetic pathways, the photoautotrophic microalgae convert the captured light and CO_2 into energy [4,33,37]. Heterotrophic microalgae grow by fermentation on organic substrates without the presence of light [13]. Some species can be grown mixotrophically, which refers to their ability to perform photosynthesis and fermentation on organic carbon sources simultaneously [32].

Eukaryotic photoautotrophic microalgae are recognized as primitive plants, as their general cell structure is comparable with plant cells. A

schematic overview of their cell structure is presented in Figure 1.2a. An often rigid cell wall surrounds the cell and forms the outside of the algal cell. The membrane located on the inner side of the cell wall functions as a selective barrier. As in plant cells, the cytosol includes several cell organelles like mitochondria, a nucleus, an endoplasmic reticulum, a vacuole, a chloroplast and a Golgi apparatus. Also lipid bodies, which store the generated energy as lipids, are present in the cytosol [34,37]. Eukaryotic heterotrophic microalgae (Figure 1.2b), are thin-walled vegetative cells containing mitochondria, a nucleus, an endoplasmic reticulum, a vacuole, a Golgi apparatus and lipid bodies [38]. The largest difference between a photoautotrophic and heterotrophic microalgal cell is thus the absence of a chloroplast in the latter. This organelle conducts photosynthesis and includes a thylakoid system rich in photosynthetic pigments, a pyrenoid and contains a part of the lipid and storage polysaccharide bodies [37]. During this PhD research, the nomenclature 'heterotrophic microalgae' was used for species that lack a chloroplast. Photoautotrophic microalgae cultivated heterotrophically or mixotrophically were not considered as heterotrophic microalgae.

The chemical composition of microalgal cells shows a big variability depending on the growth conditions such as water temperature, nutrient availability, salinity and light intensity [33]. Furthermore, the chemical composition differs depending on the species. Microalgae can be found with a wide range of protein (20-71%), carbohydrate (8-64%) and lipid (2-70%) concentrations [39]. Microalgae growing under normal conditions produce significant amounts of lipids, neutral (non-polar simple lipids such as triacylglycerols and free fatty acids) as well as polar lipids (polar more complex lipids such as phospholipids and glycolipids), in the stationary growth phase [40]. Most of the polar lipids are located in the cell membrane and the cell organelle membranes (e.g. thylakoid membrane), while the neutral lipids function as storage lipids and are more present in lipid bodies [37,41]**.** Many microalgal species are primary producers of n-3 LC-PUFA, such as EPA and DHA, in the marine food chain [42]. These fatty acids regulate the fluidity and membrane function in the microalgal cell [35,43,44]. Importantly, photoautotrophic microalgae contain a significant

amount of their EPA and DHA in the polar lipids, which implies that they are located amongst others in the thylakoid membrane, and thus in the proximity of the photosynthetic pigments [45–47].

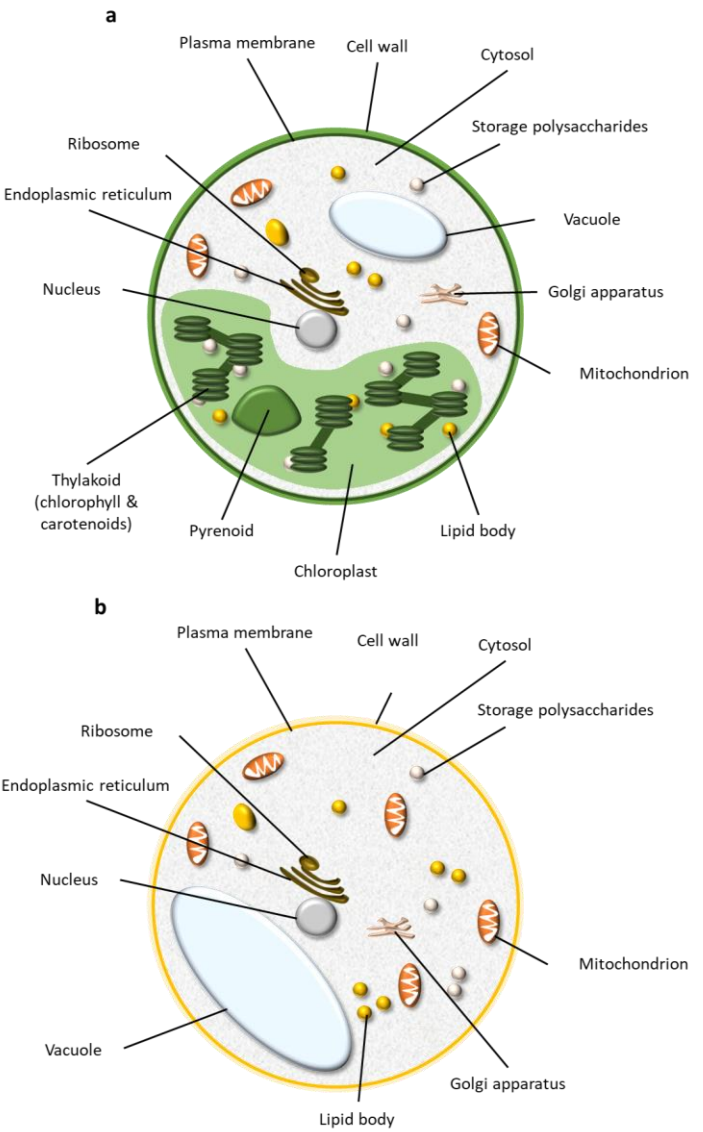


Figure 1.2. *Schematic overview of an eukaryotic photoautotrophic microalgal cell (a) and an eukaryotic heterotrophic microalgal cell (b), based on Pignolet et al. [37], Baudelet et al. [48], Poojary et al. [49] and Yokoyama et al. [38]. Some organelles might be absent or differently organized in certain microalgal species.*

Table 1.1 reports the EPA and DHA concentrations (as % of total fatty acids) of different microalgal species (based on Ryckebosch *et al.* [4]).

These high amounts of n-3 LC-PUFA make microalgae a reliable alternative source for fish and fish oil. Furthermore, microalgae have different advantages compared to fish as traditional n-3 LC-PUFA source. Microalgal organisms grow exponentially and this high growth rate allows to collect large amounts of biomass in a limited time. They are cultivated in reactors or open pond systems, of which the conditions can be controlled, leading to a more constant biochemical composition and the absence of chemical contaminants. Moreover, no fertile land is needed and microalgae fit in a vegetarian, and even vegan, diet [4]. Besides, EPA and DHA of photoautotrophic microalgae are often incorporated in the polar lipids, which may lead to a higher bioavailability compared to n-3 LC-PUFA in the neutral lipids [45–47]. Moreover, a higher oxidative stability was observed in photoautotrophic microalgal oil, which is probably explained by the presence of endogenous antioxidants [44,50].

Table 1.1. *Overview of total lipids (%), EPA and DHA concentrations (as % of total fatty acids) in different microalgal species (based on Ryckebosch et al. [4])*

Phylum	Species	Total lipids	EPA	DHA
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
	Isochrysis	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

1.3. PRODUCTION CHAIN OF MICROALGAE

Before high quality n-3 LC-PUFA rich microalgal biomass can be used in food applications, different steps in the production process should be completed (Figure 1.3) [51].

Photoautotrophic microalgae are mainly cultivated in open raceway ponds or in closed photobioreactors. A lower operational cost and an easier handling while upscaling the production characterize open raceway ponds. However, they are less controlled and therewith less resistant to biological contamination compared to closed photobioreactors. Usually, a cell density of 0.5 g/L in open pond reactors and up to 5 g/L in closed photobioreactors can be obtained [52,53]. Heterotrophic microalgae are typically grown in a conventional fermentor, in which a cell density of 25-150 g/L can be achieved [54,55]. The cultivation curve of microalgae proceeds according to a fixed pattern. First, microalgae need some time to adapt to their new conditions. In this phase, called the lag phase, there is almost no growth. This period of adaptation is followed by the exponential phase, where microalgae multiply rapidly and the cell density increases [40,56]. In the stationary phase, nutrients become limited causing a smaller cell density increase [57]. Some studies have reported the stationary phase as the favored phase to obtain the highest concentrations of EPA and DHA (as % of total fatty acids) [58,59], while others have reported conflicting results by the determinination of higher amounts in the exponential phase [60].

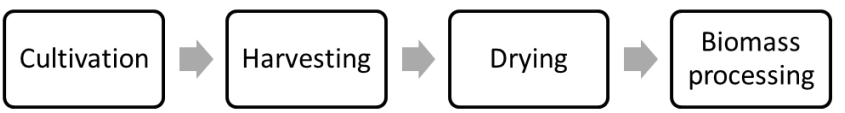


Figure 1.3. *Production process of microalgae and their derived products (based on Vandamme [51]).*

During the harvesting process, a large volume of water, originating from the growth medium, is removed to obtain a wet microalgal paste [61]. Different harvesting techniques can be used, amongst others

centrifugation, filtration, flotation, flocculation and sedimentation [53]. Centrifugation is the common harvesting method since it is rapid, efficient and suitable for most species. On the other hand, it is an expensive and energy-intensive way to separate the microalgae from their growth medium. Therefore in the past years, more research on alternative and low cost harvesting methods is being performed [62–64].

The paste obtained by harvesting (15-25% dry weight) is susceptible to lipolytic degradation by endogenous hydrolytic enzymes. Balduyck *et al.* [65] observed that free fatty acids are formed very rapidly during post-harvest storage of wet biomass of *Isochrysis* at 20°C and 4°C. The lipolysis rate is however strongly influenced by the cell integrity, depending on species and pretreatment [66]. Free fatty acids oxidize more easily compared to esterified fatty acids [67] and therefore a supplemental drying process is performed to obtain stable and dry microalgal biomass. Additionally, a higher microbial stability is obtained by drying the biomass. Commonly, freeze drying or spray drying are the techniques used [68,69].

Once dried, the microalgal biomass can be incorporated into the food chain in different ways. Three different dosage forms can be distinguished: direct incorporation of the whole biomass, direct incorporation of oil extracted from the biomass and indirect application as an animal feed supplement [33]. When incorporating the biomass, no further downstream processing is needed. For the incorporation of n-3 LC-PUFA oil, a supplemental extraction step has to be executed [70]. Both, microalgal biomass and oil, can be used as an animal feed supplement as well. This may lead to an indirect increase of n-3 LC-PUFA in the food chain via animal derived products like eggs or meat [71,72]. Indirect incorporation will not be discussed further in this chapter.

1.4. DIRECT INCORPORATION OF N-3 LC-PUFA RICH MICROALGAE INTO THE FOOD CHAIN

During the past decade, there has been an increased interest to enrich food products with n-3 LC-PUFA, since no change in eating habits is then needed to enhance their intake and as such a larger part of the population is attained [12]. Research has been performed on the enrichment of food products with n-3 LC-PUFA derived from microalgae. Table 1.2 presents an overview of the literature on the direct incorporation of n-3 LC-PUFA derived from microalgal species in food products. This overview summarizes the studies, chronologically per microalgal species, focusing on n-3 LC-PUFA rich microalgal species cultivated both photoautotrophically and heterotrophically. A clear difference in the used delivery system of photoautotrophic and heterotrophic microalgae can be observed. All photoautotrophic microalgae were incorporated as intact biomass, while heterotrophic microalgae were incorporated as oil. Only biomass of *Tetraselmis* and oil extracted from *Schizochytrium* and *Crypthecodinium* are labeled as Generally Recognized As Save (GRAS) by the Food and Drug Administration (FDA) and food grade by EFSA. This explains the focus on the incorporation of photoautotrophic biomass on the one hand and heterotrophic microalgal oil on the other hand [24,73]. The research on the incorporation of photoautotrophic and heterotrophic microalgae targets different food products. The focus for photoautotrophic biomass is mainly on biscuits and other cereal products, in contrast to heterotrophic oil for which egg, meat and fish products are the main products of interest. The amount of added biomass or oil and the final concentration of n-3 LC-PUFA in the food product is presented (Table 1.2) as well. These numbers were provided by the authors or calculated, if possible. A large variation of n-3 LC-PUFA addition can be observed. The claim 'rich in omega-3' on a food product demands at least 80 mg of EPA + DHA per 100 g or per 100 kcal, whichever is limiting [74]. The incorporation of the heterotrophic oil is often overdosed compared to the n-3 LC-PUFA claim. For each of the studies the focus of the experiments is indicated as well in the table. For photoautotrophic microalgae, it can be

observed that besides the amount of n-3 LC-PUFA, mainly sensorial characteristics, texture and color were evaluated. The number of studies focusing on the impact of food processing steps and lipid oxidation are very limited. The opposite could be observed for studies using heterotrophic oil, where a strong focus on lipid oxidation is observed. Sensorial characteristics, texture and color were also often taken into account. Furthermore, it was observed that a limited number of research groups executes the studies dealing with the incorporation of photoautotrophic biomass on the one hand and heterotrophic oil on the other. The results of the different studies will be discussed per microalgal species in detail below.

1.4.1. PHOTOAUTOTROPHIC MICROALGAE

1.4.1.1. *DIACRONEMA* SP.

The brownish-green microalga *Diacronema* sp., formerly called *Pavlova* and taxonomically belonging to *Haptophyceae*, is a marine microalga containing EPA and DHA [75]. The potential of this species was screened in gelled desserts as a dairy analog and pasta by Gouveia *et al.* [76] and Fradique *et al.* [77], respectively.

i. *N-3 LC-PUFA AND PROCESSING*

The authors of both studies incorporated similar amounts of *Diacronema* biomass in concentrations varying between 0.1 and 2%. As expected, this led to an increase in n-3 LC-PUFA compared to the control in all samples [76,77]. Fradique *et al.* [77] reported 20, 40 and 80 mg n-3 LC-PUFA/100 g pasta by the addition of respectively 0.5, 1 and 2% microalgal biomass. These amounts are in line with regulations to claim the product as a source of n-3 LC-PUFA (40 mg) or rich in n-3 LC-PUFA (80 mg) [74]. Gouveia *et al.* [76] only presented relative amounts of EPA and DHA, which were not sufficient to calculate absolute amounts of n-3 LC-PUFA in the final product.

Table 1.2. *Overview of research on the direct incorporation of n-3 LC-PUFA derived from microalgae in food products*

Growth	Delivery system	Microalgal species	Food product	Amount of addition (%)	Amount of n-3 LC-PUFA (mg/100 g)	Focus on					References
						n-3 LC-PUFA	Processing	Lipid Oxidation	Sensorial characteristic	Texture	Color	
Photoautotrophic	Biomass	*Diacronema* sp.	Dairy analogs	0.1/0.25/ 0.5/0.75		x	x			x	x	Gouveia et al. [76]
			Pasta	0.5/1/2	20/40/80	x	x		x	x	x	Fradique et al. [77]
		Isochrysis sp.	Biscuits	1/3	101/310	x	x			x	x	Gouveia et al. [82]
			Pasta	0.5/1/2	30/60/120	x			x			Fradique et al. [77]
			Chikkis	1/3	102/320	x		x	x	x	x	Babuskin et al. [83]
			Bread	0.4						x	x	Garcia-Segovia et al. [84]
		Nannochloropsis sp.	Pasta Biscuits	0.5/1/3 1/2/3	31/63/190 98/197/298	x				x	x	Babuskin et al. [87]
			Pasta	3/6/9/12	39/128/237 /310	x			x	x		Rodriguez De Marco et al. [88]
			Chikkis	1/3	75/240	x		x	x	x	x	Babuskin et al. [83]
			Bread	0.4						x	x	Garcia-Segovia et al. [84]
		Phaeodactylum sp.	Biscuits	2/6						x	x	Batista et al. [90]
		Tetraselmis sp.	Bread	0.4						x	x	Garcia-Segovia et al. [84]

Growth	Delivery system	Microalgal species	Food product	Amount of addition (%)	Amount of n-3 LC-PUFA (mg/100 g)	Focus on						References
						n-3 LC-PUFA	Processing	Lipid Oxidation	Sensorial characteristic	Texture	Color	
Heterotrophic	Oil	*Crypthecodinium* sp.	Surimi		588	x		x		x	x	Park et al.[92]
			Yoghurt	0.18	147			x	x			Chee et al. [93]
			Milk	2/5/10	800/2000/ 4000			x	x			Gallaher et al. [101]
			Patties, sausages, ham		454	x	x	x	x		x	Lee et al. [94]
			Sausages	1.1	400	x						Lopez-Lopez et al. [95]
			Sausages	10	3500	x		x	x		x	García-Íñiguez de Ciriano et al., [96]
			Egg sticks	10	4000	x						Kassis et al. [97]
			Surimi		588	x		x		x	x	Pietrowski et al. [98]
			Egg sticks	10	4000			x	x	x	x	Sedoski et al. [99]
			Patties	0.1	24.4	x	x	x	x			Alejandre et al. [100]

Growth	Delivery system	Microalgal species	Food product	Amount of addition (%)	Amount of n-3 LC-PUFA (mg/100 g product)	Focus on						References
						n-3 LC-PUFA	Processing	Lipid Oxidation	Sensorial characteristic	Texture	Color	
Heterotrophic	Oil	*Schizochytrium* sp.	Bread	0.325/ 0.65	78/156	x			x	x		Serna-Saldivar et al. [104]
			Sausages	15/25	1340/1490	x		x	x			Valencia et al. [105]
			Surimi	0.5/1	200/400	x		x		x		Tolasa et al. [106]
			Sausages	1.32	450	x		x	x	x	x	Berasategi et al. [107]
			Egg sticks	0.35	140	x		x	x		x	Lamas et al. [108]

The authors of both studies reported no significant impact of cooking the pasta (3-5 min in boiling water) or heating the dessert to gelling temperatures (5 min at 90°C) on the n-3 LC-PUFA concentration. This indicates a thermal resistance of EPA and DHA in *Diacronema* biomass during processing of these food products [76,77].

ii. *LIPID OXIDATION*

No lipid oxidation parameters were followed in the studies.

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

At levels of 80 mg n-3 LC-PUFA addition (2% biomass) per 100 g pasta, an untrained panel perceived a depreciative fish flavor. They marked the strange flavor with 7 on a scale of 9 compared to a mark of 2 for the control and incorporation concentrations of 20 mg n-3 LC-PUFA/100 g (0.5% biomass). Furthermore, on the global appreciation the panel showed a preference for the control pasta (almost 7 on a scale of 10), although the enriched pasta (0.5 and 1%) was positively classified (5.5 on a scale of 10). The highest incorporation concentration (2%) was marked with a score of 3 and was thus unacceptable [77].

The texture of the pasta was not influenced by the incorporation of the microalgal biomass. The same mark of almost 7 (on a scale of 10) was given for the enriched pasta as well as for the control [77]. On the contrary, the gelled dessert reinforced by addition of higher incorporation concentrations, which is undesirable for such food products [76].

The score for the color did not depend on incorporation concentration, but was always lower compared to the control [76,77].

1.4.1.2. *ISOCHRYSIS* SP.

Isochrysis sp., with some strains currently named as T-*Isochrysis*, is a brownish-golden marine microalga containing EPA and DHA [78,79]. Normally the presence of EPA in the fatty acid profile is rather limited and thus larger amounts of EPA probably indicate contamination during cultivation [80,81]. This species belongs to the phylum *Haptophyta.* The

potential of this species was screened in biscuits, pasta, chikkis (a traditional Indian sweet generally made from groundnuts and jaggery) and bread in studies of Gouveia *et al.* [82], Fradique *et al.* [77], Babuskin *et al.* [83] and Garcia-Segovia *et al.* [84].

i. *N-3 LC-PUFA AND PROCESSING*

The microalgal biomass of *Isochrysis* was incorporated in the food products in concentrations between 0.4 and 3% [77,82–84] logically leading to an increased n-3 LC-PUFA content compared to the control varying between 30 and 310 mg/100 g product [77,82,83]. In the studies of Gouveia *et al.* [82], Fradique *et al.* [77] and Babuskin *et al.* [83] the addition of 1% biomass resulted in different amounts of n-3 LC-PUFA in the final product, respectively 101 mg n-3 LC-PUFA/100 g biscuits, 60 mg n-3 LC-PUFA/100 g pasta and 102 mg n-3 LC-PUFA/100 g chikkis. The observed differences may be explained by the different ways to prepare the food products and batch variation and thus the composition of the used microalgae. Garcia-Segovia *et al.* [84] presented no results about the fatty acid profile of the microalgae nor the amount of n-3 LC-PUFA in the final food product.

Gouveia *et al.* [82] only determined the n-3 LC-PUFA content after the baking process (30 min at 180°C). As expected, the amount of n-3 LC-PUFA was 3 times higher in the biscuits with 3% incorporated compared to 1%. Based on these results the authors suggested that the encapsulation of the n-3 LC-PUFA by the cells could protect them against thermal degradation. However, the amount of n-3 LC-PUFA of the biscuits before the baking process was not determined.

ii. *LIPID OXIDATION*

Babuskin *et al.* [83] stored the chikkis enriched with 3% biomass for 60 days at 27°C. Natural (rosemary extract) or synthetic (butylated hydroxyanisol (BHA)) antioxidants were added in different concentrations (1-5%). The peroxide value of the control (3% biomass without supplemental antioxidants) increased significantly after 30 days of storage, while the value of the chikkis with additional antioxidants did not. The peroxide value was determined by an iodometric titration, which is not the optimal

method in colored food products enriched with photoautotrophic microalgae. This is discussed in more detail in chapter 2 and 3. Besides, it is suggested to follow both primary as well as secondary oxidation. The authors concluded that these results demonstrate the need for additional antioxidants in the food product, although the increase in the peroxide value of the control (between zero and 60 days) is still reasonably low (10 meq hydroperoxides/kg oil) taking into account normal peroxide value interferences. The obtained values were also comparable with the results of Ryckebosch *et al.* [50], who stored *Isochrysis* oil for 8 weeks at 37°C. Ryckebosch *et al.* [50] observed, in contrast, an increase in the peroxide values for commercial n-3 LC-PUFA sources up to 150 meq hydroperoxides/kg oil (between zero and 14 days).

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

Similar results for the sensorial characteristics were observed for pasta and chikkis enriched with n-3 LC-PUFA derived from *Isochrysis* in the study of Fradique *et al.* [77] and Babuskin *et al.* [83] respectively. Food products without microalgae were preferred compared to those enriched with microalgae, although the panelists have classified the enriched products as 'acceptable'. Moreover, the incorporation concentration had a significant impact on the global appreciation. Pasta and chikkis enriched with 1% microalgal biomass were marked with 6 and 7.1, while the control obtained respectively 7 and 8.2. Products enriched with a higher concentration (up to 3%) showed a lower global appreciation of 4 and 4.9, because a fishy flavor was observed at these higher incorporation concentrations.

The texture of pasta, chikkis and bread was not modified by the incorporation of *Isochrysis* biomass compared to the control, while the firmness of the biscuits increased linearly with the microalgal biomass concentration [77,82–84]. The authors suggested that the incorporation of microalgal biomass might have an impact on the water absorption process, which has an influence on the firmness. The impact depends on the type of food product, whether or not a small amount of flour was

replaced by the microalgal biomass, incorporation concentration, etc. [82,84].

The color of the enriched products was scored lower compared to the control and was limitedly influenced by the incorporation concentration. Decreased L* (meaning darker), a* (meaning more red and less green) and b* (meaning more blue and less yellow) values were observed, which can be ascribed to the pigments of the microalgal biomass [77,82–84]. In the studies of Gouveia *et al.* [82] and Babuskin *et al.* [83] enriched food products were stored for respectively 12 and 8 weeks and within a sample no color changes could be detected over this time frame.

1.4.1.3. *NANNOCHLOROPSIS* SP.

The greenish, marine microalga *Nannochloropsis* sp., belonging to the *Haptophyceae* and also named as *Microchloropsis*, contains high amounts of EPA [85,86]. Its potential was screened in chikkis, pasta, biscuits and bread by Babuskin *et al.* [83,87], Rodriguez De Marco *et al.* [88] and Garcia-Segovia *et al.* [84].

i. *N-3 LC-PUFA AND PROCESSING*

Food products were enriched with 0.4 to 12% of *Nannochloropsis* biomass [83,84,87,88]. Babuskin *et al.* [83,87] observed an n-3 LC-PUFA concentration of 63, 75 and 198 mg/100 g product for respectively pasta, chikkis and biscuits enriched with 1% biomass and 190, 240 and 298 mg/100 g product for an incorporation concentration of 3%, while no n-3 LC-PUFA was present in the control. Rodriguez De Marco *et al.* [88] observed 39, 128, 237 and 310 mg n-3 LC-PUFA/100 g pasta for incorporation concentration of 3, 6, 9 and 12% respectively. Obviously, higher incorporation concentrations resulted in linearly higher levels of n-3 LC-PUFA in the final product. Pasta, chikkis and biscuits were prepared in a different way, which can explain the differences of the n-3 LC-PUFA concentration in the final product when adding the same amount of biomass (1 or 3%). Besides another batch of *Nannochloropsis* sp. may have a large influence on the n-3 LC-PUFA concentration of the biomass

and thus also on the n-3 LC-PUFA concentration of the final product. No results about the fatty acid profile of the enriched bread were provided [84].

None of the studies focused on the impact of processing on the n-3 LC-PUFA concentration in the product.

ii. *LIPID OXIDATION*

A 60 days storage experiment executed by Babuskin *et al.* [83] at 27°C with 3% *Nannochloropsis* enriched chikkis without and with added (natural or synthetic) antioxidants resulted in an increase of the peroxide value after 15 days for all samples. The control (without added antioxidants) showed the highest increase in the peroxide value (10 meq hydroperoxides/kg oil) between zero and 60 days, while for the chikkis with antioxidants an increase between 1.5 and 5 meq hydroperoxides/kg oil was observed. As already explained above for *Isochrysis*, the authors stated, based on these results, that extra antioxidants were needed for the prevention of lipid oxidation in enriched chikkis. However, the increase in the peroxide value is still low compared with the results of the commercial n-3 LC-PUFA sources of Ryckebosch *et al.* [50].

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

The sensorial characteristics of enriched chikkis, pasta and cookies showed similar results in the studies of Babuskin *et al.* [83,87]. The overall acceptability of food products without incorporation was higher with a mark of respectively 7.9, 7.9 and 7 on a scale of 9. However, incorporation concentrations up to 2% for cookies and up to 3% for chikkis and pasta were still positively classified (i.e., with a mark higher than 4.5), while a fishy taste was observed by the panelist in cookies with an incorporation concentration of 3% probably explaining the lower global appreciation (3.3) of these products. In the study of Rodriguez De Marco *et al.* [88], pasta enriched with 3% *Nannochloropsis* was still positively classified by the consumers panel with a score of 6.1 on a scale of 9 (compared to a score of 7.2 for the control). The higher incorporation concentrations decreased the global acceptance of the pasta. The 9% incorporation concentration was not acceptable anymore, with a score of 4.1.

The firmness of the cookies and pasta increased with a higher incorporation concentration, while the texture of chikkis and bread was not affected by the used incorporation concentrations [83,84,87,88]. As already explained above for *Isochrysis*, possibly the incorporation of microalgal biomass may have an impact on the water absorption process. Textural changes during storage within a sample were comparable for all enriched samples as well as for the control [83,87].

The color of the food products (chikkis, pasta, biscuits and bread) changed by incorporation of *Nannochloropsis*. The lightness value L* for these products decreased with a factor 1.2 to 1.7 compared to the control, meaning the enriched products were darker. In none of the products, the L* value was influenced by the incorporation concentration. The a* value decreased dramatically (less than half of the value of the control) in all enriched food products and a larger decrease was observed at higher incorporation concentrations. The b* value was only limitedly influenced by the addition of *Nannochloropsis.* In pasta a slight increase of the b* value could be observed, while in bread no significant difference and in chikkis and cookies a slight decrease was detected [83,84,87]. Furthermore, no significant impact of the incorporation concentration could be detected. In the studies of Babuskin *et al.* [83,87] storage of the incorporated food products for 8 weeks showed no color changes over time.

1.4.1.4. *PHAEODACTYLUM* SP.

Phaeodactylum sp. is a brown-greenish marine diatom belonging to the *Bacillariophyceae*. This microalgae contains high amounts of EPA [89]. Batista *et al.* [90] screened its potential incorporation in biscuits.

i. *N-3 LC-PUFA AND PROCESSING*

Although n-3 LC-PUFA rich *Phaeodactylum* was incorporated, no efforts were made to obtain data for the n-3 LC-PUFA content of biscuits with 2 and 6% added biomass.

ii. *LIPID OXIDATION*

No lipid oxidation parameters were followed in the study either.

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

In this study, the texture of biscuits enriched with 2 and 6% *Phaeodactylum* biomass was investigated. The hardness did not differ significantly between the biscuits with 2% addition and the control, while the hardness was significantly higher for the biscuits with 6% addition. The authors suggested that when microalgae were added to the cookie dough, they absorbed more water, which reinforced the cookie internal structure. The hardness of each biscuit did not change significantly during 8 weeks of storage [90].

Lower L* (50 and 35) and a* (-4 and -3) and higher b* (35 and 30) values were detected for biscuits enriched with 2 and 6% biomass, respectively, compared to the control (80, -1 and 25). This can be related to the high amounts of the carotenoid fucoxanthin present in the *Phaeodactylum* biomass. A total color difference (ΔE*) of 7.63, 18.97 and 23.63 for the baked control biscuit and those enriched with 2 and 6% biomass, respectively, was observed compared to the raw control. During an 8 week storage period, the total color differences within a sample were lower than 5, which is not visible by the human eye [90].

1.4.1.5. *TETRASELMIS* SP.

Tetraselmis sp. is a green microalga belonging to the *Chlorophyta*. Mainly EPA as n-3 LC-PUFA source can be found in this microalgal species. The total amount of n-3 LC-PUFA is rather limited, which can explain the limited amount of studies focusing on *Tetraselmis* as n-3 LC-PUFA source. The potential use of *Tetraselmis* sp. was screened in bread by Garcia-Segovia *et al.* [84].

i. *N-3 LC-PUFA AND PROCESSING*

Garcia-Segovia *et al.* [84] incorporated 0.4% *Tetraselmis* in bread, but no further results on the n-3 LC-PUFA incorporation were shown.

ii. *LIPID OXIDATION*

No lipid oxidation parameters were followed in this study either.

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

Garcia-Segovia *et al.* [84] screened the influence of 0.4% *Tetraselmis* biomass incorporation on the texture and color parameters of bread. The texture was not significantly modified by the added microalgal biomass.

On the contrary, incorporation of *Tetraselmis* did influence the color. A decrease in the lightness (L* value) from 73 to 62 was observed compared to the control. The redness (a* value) increased from 2.72 to 3.08 compared to the control, while the yellowness (b* value) remained unmodified [84].

1.4.1.6. GENERAL CONCLUSION

The potential of different photoautotrophic microalgae (*Diacronema, Isochrysis, Nannochloropsis, Phaeodactylum* and *Tetraselmis*) has already been screened in food products [76,77,82–84,87,88,90]. However, the range of food products is mostly limited to biscuits and other cereal products**.** Therefore, it would be interesting to gain more insight into a broader range of food products. All studies described above have used photoautotrophic microalgae as an alternative source of n-3 LC-PUFA, but some of them have not measured the n-3 LC-PUFA concentration of the enriched food product. Those who did, showed that the addition of microalgae is indeed an effective way to increase the n-3 LC-PUFA concentration in the food products. Incorporation concentrations up to 3% (*Diacronema, Isochrysis, Nannochloropsis* and *Phaeodactylum)* were in most cases sufficient to claim the products as 'a source of' or 'rich in' n-3 LC-PUFA. Processing may have an impact on the final n-3 LC-PUFA concentration given mechanical and thermal process steps are often exposed at high pressure and temperature, but unfortunately, this has rarely been taken into account. Gouveia *et al.* [76,82] and Fradique *et al.* [77] found no significant impact of food processing steps (boiling in water or oven baking) on the n-3 LC-PUFA concentration.

The oxidative stability is also an important quality parameter of the final product given the proneness of n-3 LC-PUFA to oxidation, but only Babuskin *et al.* [83] have paid attention to it. Both, incorporation of

Isochrysis and *Nannochloropsis* in chikkis resulted in an increase of the peroxide value with 10 meq hydroperoxides/kg oil during 2 months of storage at 27°C. These values are still reasonable low compared to literature, but the authors observed that the addition of supplemental antioxidants might increase the oxidative stability.

The sensorial characteristics of the enriched food products were marked lower than the control in all studies, but were in most cases still classified positively [77,83,87]. At higher concentrations (2% in biscuits and 3% in pasta and chikkis), the panelists observed a fishy taste, which explains the lower global appreciation of these food products. Similar results for the sensorial characteristics were found for the different microalgal species and the different food products. The firmness of cookies and pasta increased with a higher incorporation concentration, while the texture of chikkis and bread was not affected by the incorporation concentration. Again, no impact of the used species could be detected. Some authors followed textural changes over time, but in all cases, comparable results were obtained as for the control. The color of the food products changed by incorporation of the pigment rich photoautotrophic microalgae. In general the lightness value L* and the redness value a* decreased, while the yellowness b* value was only limitedly influenced. Studies following the color of the enriched products over time showed no changes [82,83,87,90].

1.4.2. HETEROTROPHIC MICROALGAE

1.4.2.1. *CRYPTHECODINIUM* SP.

The microalga *Crypthecodinium* sp. is a heterotrophic microalgae belonging to the *Dinophyceae* and is rich in the fatty acid DHA [91]. The incorporation of *Crypthecodinium* oil was screened in dairy, egg sticks (liquid egg products uniformly cooked using a microwave oven to gelled products) and fish and meat products [92–101].

i. *N-3 LC-PUFA AND PROCESSING*

A variety of food products was enriched with 0.1 to 10% *Crypthecodinium* oil, resulting in n-3 LC-PUFA concentrations of 24.4 to 4000 mg/100 g

products. The highest amounts are far above the minimum required for an EU nutrition claim [74]. Park *et al.* [92] and Pietrowski *et al.* [98] enriched surimi with 588 mg n-3 LC-PUFA/100 g. The incorporation concentration in yogurt (0.18% addition) resulted in 147 mg/100 g, while for milk (2-10% addition) amounts between 800 and 4000 mg/100 g were obtained [94,101]. Kassis *et al.* [97] and Sedoski *et al.* [99] enriched egg sticks with 10% heterotrophic microalgal oil to obtain 4000 mg/100 g egg product. Lee *et al.* [94], Lopez-Lopez *et al.* [95], García-Íñiguez de Ciriano *et al.* [96] and Alejandre *et al.* [100] incorporated *Crypthecodinium* oil in patties, sausages and ham. The patties were enriched with 24.4 up to 454 mg/100 g product, while the sausages contained 400 up to 3500 mg/100 g final product and ham 454 mg/100 g product. As expected, higher n-3 LC-PUFA concentrations were obtained with higher incorporation concentrations.

Only Lee *et al.* [94] and Alejandre *et al.* [100] studied the impact of processing. Lee *et al.* [94] observed losses of n-3 LC-PUFA up to 20% due to cooking the patties, sausages or ham, while in the study of Alejandre *et al.* [100] the cooking process did not affect the n-3 LC-PUFA concentration in the patties. The different cooking temperature and time during processing are a possible explanation for the observed differences.

ii. *LIPID OXIDATION*

The oxidative stability of surimi was investigated by Park *et al.* [92] and Pietrowski *et al.* [98]. Park *et al.* [92] observed an impact of the delivery system of the microalgal n-3 LC-PUFA. During 27 days storage at 6°C, lipid oxidation increased twice as much when the n-3 LC-PUFA was added as emulsified algal oil compared to bulk oil, in both cases without the addition of antioxidants. Addition of cryoprotectants or antioxidants reduced the lipid oxidation, no differences between the enriched samples themselves as well as with the control could be observed anymore. Pietrowski *et al.* [98] determined thiobarbituric acid reactive substances (TBARS) values (as a marker for secondary oxidation products) after preparing the surimi gels, but did not store the samples longer to follow up further oxidation. An increase of thiobarbituric acid reactive substances (TBARS) value from 0.6 to 0.8 mg malondialdehyde/kg sample

was observed by the incorporation of *Crypthecodinium* oil compared to the control. Based on both studies it seems that surimi gel enriched with *Crypthecodinium* oil was not oxidatively stable without the addition of antioxidants as Ke *et al.* [102] proposed that TBARS values for seafood products above 0.58 mg malondialdehyde/kg sample were slightly oxidized and rancid. Gallaher *et al.* [101] and Chee *et al.* [93] investigated oxidation in enriched dairy products. Gallaher *et al.* [101] could not detect an increase of hydroperoxides (as a marker for primary oxidation) nor propanal (as a marker for secondary oxidation) in any enriched milk, while water supplemented with the same amount of *Crypthecodinium* oil started to oxidize. The authors ascribed the antioxidant properties of milk to the casein. It has to be remarked however that 24 hours at 32°C is a short storage period to draw conclusions on oxidative stability. In contrast, hydroperoxides were formed in n-3 LC-PUFA enriched strawberry flavored yogurt during 21 days of storage at 4°C [93]. Logically, the control without large amounts of unsaturated fatty acids did not oxidize. Based on the results, the authors suggested the need for the addition of antioxidants in enriched food products although the total amount of lipid hydroperoxides was limited to 3 meq hydroperoxides/kg oil after 21 days of storage. Lee *et al.* [94] determined the oxidative stability of n-3 LC-PUFA incorporated ham with and without added antioxidants during storage for 21 days under vacuum at -18 and 4°C. A limited increase in the peroxide value from day zero (non-detectable) to day 21 (7.11 and 5.22 meq hydroperoxide/kg sample), when stored at -18 and 4°C respectively, could be detected in samples without added antioxidants. However, adding a cocktail of antioxidants prevented the products form oxidation. García-Íñiguez de Ciriano *et al.* [96] evaluated the oxidative stability of sausages during 30 days of ripening at an unspecified temperature. The TBARS values of the enriched samples as well as of the control were very low (below 0.45 mg malondialdehyde/kg oil) with no significant changes over time, but again antioxidants had been added to control potential oxidation. Alejandre *et al.* [100] stored enriched patties vacuum and non-vacuum at 4 and 25°C for 31 days. Vacuum-packed patties stored at 4 and 25°C and non-vacuum packed patties stored at 4°C

remained stable during storage. Patties packed non-vacuum and stored at 25°C showed a significant increase in TBARS (from 12.45 to 30.56 mg malondialdehyde/kg oil). These results again showed that it could be interesting to add some extra antioxidants in order to control lipid oxidation. TBARS values of egg sticks enriched with 4000 mg n-3 LC-PUFA/100 g were not influenced during a 14-days storage at 4°C. This result was however not surprising since the egg sticks were stored under vacuum conditions [99]. It can be concluded that most studies obtained similar results: when storing under non-vacuum conditions additional antioxidants should be added to prevent oxidation.

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

Similar sensorial characteristics were described in different studies [93,94,96,99,101,103]. For both dairy products, milk and yogurt, the panelists were not able to observe any differences between the control and the enriched food products at day zero [93,101]. However, after 15 and 21 days of storage, the sensory panel observed a fishy taste in the enriched products. Enriched meat products, except for hams, were not significantly different compared to their control [94,96,103]. The enriched hams were marked with 6.4 on a 9-point scale instead of 6.9 for the control [94]. Furthermore, 55% of the consumers gave a positive answer to the question if they would consume the enriched products [100]. The n-3 LC-PUFA enriched egg sticks (6.0 on a 9-point scale) were accepted by the consumers to the same extent as the control egg sticks (5.9) [99].

Park *et al.* [92] observed that no differences in gel strength were observed between the control (176 g cm) and surimi gel enriched with emulsified algal oil, while incorporation of bulk oil resulted in a decrease of the gel strength (116 g cm). On the contrary, Pietrowski *et al.* [98] observed no differences in springiness, cohesiveness, chewiness and resilience compared to the control. The texture of the control egg sticks was more favorably marked (6.3 on a 9-point scale) compared to the enriched sticks (5.2) [99].

The color of the egg sticks in the study of Sedoski *et al.* [99] was not significantly different compared to the control, while in surimi the b* value increased compared to the control (from 5 to 12 and from 3.72 to 27 respectively) [92,98]. However in the latter studies the L* and a* values were unaffected as well. In hams, the L* value increased slightly compared to the control (from 65 to 70), while the a* value was unaffected and no results about the b* value were described [94]. On the other hand, the L*, a* and b* values were unmodified in fermented sausages [96].

1.4.2.2. *SCHIZOCHYTRIUM* SP.

The microalga *Schizochytrium* sp. is a heterotrophic microalga belonging to the *Labyrinthulomycetes* and is rich in the fatty acid DHA [91]. The incorporation of *Schizochytrium* was screened in bread, dairy, egg sticks, fish and meat products [104–108].

i. *N-3 LC-PUFA AND PROCESSING*

Different products were enriched with low amounts (0.325 to 1.32%) of *Schizochytrium* oil, which did however result in relevant amounts of n-3 LC-PUFA in the final product (78-450 mg/100 g product) [104–106,108]. Only Valencia *et al.* [105] used amounts up to 1490 mg/100 g product, which is far above the amount needed for the food claim 'rich in n-3 LC-PUFA'. Obviously, higher incorporation concentrations resulted in a linearly higher n-3 LC-PUFA concentration in the final product, independent of the type of product [104–106]. None of the studies has taken the impact of processing into account.

ii. *LIPID OXIDATION*

Valencia *et al.* [105] stored enriched fermented sausages for 90 days at 4°C vacuum as well as non-vacuum packed, but added antioxidants (Butylated hydroxytoluene (BHT) or BHA). Berasategi *et al.* [107] stored enriched sausages for 32 days at 4°C. Again, antioxidants (*M. officinalis* extract) were added. In both studies, no sign of oxidation was observed in the enriched products. Tolasa *et al.* [106] observed an impact of the quality of

the surimi on the oxidative stability, during 4 months of storage at -18 and 3°C. Remarkable is that all samples were stored under vacuum, which already helps to prevent oxidation, but that even under vacuum conditions low quality surimi started to oxidize. The peroxide value increased from 2.5 to 52 meq hydroperoxides/kg oil (from zero to 4 months), but no significant impact of the n-3 LC-PUFA concentration was observed. The peroxide value of high quality surimi on the other hand only increased from 2.5 to 5.3 meq hydroperoxides/kg oil. Frozen storage (-18°C) prevented oxidation during 4 months of storage. Lamas *et al.* [108] stored egg sticks enriched with *Schizochytrium* oil for 30 days under frozen conditions. Comparable with the results of Tolasa *et al.* [106] no oxidation could be observed.

iii. *SENSORIAL CHARACTERISTICS, TEXTURE AND COLOR*

The panelists marked the overall acceptability and flavor of bread enriched with 3% *Schizochytrium* oil the same as the control (2 on a scale between -4 and 4), at day 1 as well as day 13 [104]. Also enriched egg sticks and sausages obtained the same results by a consumer panel as the control [107,108]. Valencia *et al.* [105] found a similar result for fermented sausages enriched with 15% *Schizochytrium* oil, although the panelists indicated the taste in a triangle test significantly different for sausages enriched with 25% *Schizochytrium* oil compared to the control.

No significant differences in texture were found in enriched bread and sausages compared to the control [104,107]. However, the texture of surimi was influenced by the incorporation concentration as well as the quality of the gel. High quality surimi with an incorporation concentration of 0.5% had the highest compressive force (62.66 kg), followed by high quality surimi with 1% microalgal oil (29.50 kg) and low quality surimi with 0.5 and 1% microalgal oil (8 kg) [106].

Color was only determined in the studies of Berasategi *et al.* [107] and Lamas *et al.* [108]. The L*, a* and b* values were significantly higher in the control egg sticks (66.5, 22 and 62.5) compared to the enriched egg sticks (65, 19 and 50.8), while for sausages the opposite was observed. The enriched

sausages had similar a* values (21) as the control, but higher L* (64 instead of 60) and b* (9 instead of 5) values. The differences can be explained by the fact that in the egg sticks not only S*chizochytrium* oil was added, but also the egg yolk was removed.

1.4.2.3. GENERAL CONCLUSION

The potential of different heterotrophic oils (*Crypthecodinium* and *Schizochytrium*) was screened in bread, egg sticks, surimi, patties, ham and sausages [63–77]. The oil of both species is authorized by the EFSA and FDA [24,73]. A wide variety of incorporation concentrations (0.1 up to 10%) was used in the studies, some of them far above the amount needed for the claim 'rich in n-3 LC-PUFA'. Oil from heterotrophic sources allows to reach high amounts of n-3 LC-PUFA in the final product with low incorporation concentrations. Obviously, higher incorporation concentrations resulted in a linearly higher n-3 LC-PUFA concentration in the final product, independently of the type of product [104–106]. Following the oxidative stability of food products enriched with heterotrophic oil was often executed under vacuum and by preventively adding antioxidants. However, even under these conditions, it was not always possible to keep the enriched products oxidatively stable showing the need for preventive actions. Sensorial characteristics and texture were only seldom changed compared to the control by the incorporation of heterotrophic oil. The color was only limitedly influenced and mainly in the b* value, which expresses changes in yellowness.

1.5. COMPARISON OF PHOTOAUTOTROPHIC AND HETEROTROPHIC MICROALGAE

Both photoautotrophic and heterotrophic microalgae have shown their potential as an alternative source of n-3 LC-PUFA in different food products with each their advantages and disadvantages. Photoautotrophic biomass seem to have a higher oxidative stability than heterotrophic oil. Incorporation of heterotrophic microalgae demanded

the addition of antioxidants or vacuum packaging, which was even not always enough to maintain the oxidative stability. On the other hand, the presence of pigments in photoautotrophic biomass seem to result in larger changes in color of the enriched product compared to the heterotrophic oil. The controls showed similar sensorial characteristics as products enriched with heterotrophic microalgal oil, while the control was preferred over products enriched with photoautotrophic biomass. Although the latter were still positively classified. However, the incorporation concentration was limited to a maximum (depending on food product and species). Above this limit, a fishy taste was observed. The texture was mainly influenced by the addition of photoautotrophic biomass and was logically depending on the concentration.

Addition of microalgae as a source of n-3 LC-PUFA to food products is thus an effective way to increase n-3 LC-PUFA levels in the diet. However, comparison of the different studies was not always straightforward due to differences in set-up (food products, concentrations, storage conditions) and none of the studies used photoautotrophic as well as heterotrophic microalgae or oil in the same study. Further research on the potential of microalgae as n-3 LC-PUFA source in a broader range of food products, in standardized set-ups, paying attention to the impact of processing, etc. and development in the legislation to authorize new species, should thus be executed to support prospective applications. Therefore, the main goal of this PhD (chapters 4 – 7) was to investigate in-depth the potential of microalgae as an alternative source of n-3 LC-PUFA in vegetable-based products. The focus was especially on the impact of dosage form, microalgal species and type of vegetable puree on the n-3 LC-PUFA and endogenous antioxidant concentration of enriched vegetable purees and on the oxidative stability of enriched vegetable purees.

Chapter 2:

LIPID OXIDATION

2.1. INTRODUCTION

Unsaturated lipids, in particular those containing n-3 LC-PUFA, are highly susceptible to lipid oxidation due to their chemical structure, which includes a large number of double bounds. Lipid oxidation leads to an undesired and rancid flavor, with a low threshold value [5]. Furthermore, the nutritional value related to n-3 LC-PUFA is reduced [50] and toxic compounds, linked to the development of inflammatory diseases, cancer and atherosclerosis, can be formed [109].

Lipid oxidation of n-3 LC-PUFA plays a central role in this PhD. Therefore, this chapter summarizes the main principles of primary (section 2.2) and secondary (section 2.3) lipid oxidation. In section 2.4, the influencing factors, with special attention to the antioxidant compounds originating from microalgae and vegetables, are discussed. Finally, an overview of the most common analytical methods to monitor lipid oxidation is given in section 2.5.

2.2. PRIMARY OXIDATION

Different forms of lipid oxidation can occur: autoxidation, photo-oxidation and enzymatic oxidation. However, in each of these processes, hydroperoxides are formed as primary oxidation products. **Autoxidation** is the autocatalytic reaction of oxygen with unsaturated lipids via a free radical chain mechanism, a complex process of consecutive and overlapping reactions [5]. The primary autoxidation, in which mainly hydroperoxides are formed, consists of 3 phases: initiation, propagation and termination [110,111].

Initiation: $RH \rightarrow R^* + H^*$ (2.1)

Propagation: $R^* + O_2 \rightarrow ROO^*$ (2.2)

$ROO^* + RH \rightarrow ROOH + R^*$ (2.3)

Termination: $2\ ROO^* \rightarrow ROOR + O_2$ (2.4)

$ROO^* + R^* \rightarrow ROOR$ (2.5)

$2\ R^* \rightarrow RR$ (2.6)

The free radical chain reaction starts with the formation of a lipid radical (R*) by the abstraction of a hydrogen (reaction 2.1). Metals, increased temperature, etc. can initiate this mechanism. The propagation phase consists of two steps. First, the lipid radical reacts with triplet oxygen to form a lipid hydroperoxide radical (ROO*) (reaction 2.2), which subsequently abstracts a hydrogen from an unsaturated lipid to form a lipid hydroperoxide (ROOH) and a new lipid radical (reaction 2.3). This step is slow and rate determining. The formation of the new lipid radical explains the autocatalytic character of this reaction. The termination phase refers to the reaction of the formed radicals with each other to form a non-radical product (reaction 2.4, 2.5, 2.6), which cannot participate to the reaction anymore [5,112].

Photo-oxidation appears in the presence of light. Triplet oxygen ($^3O_2$), the ground state of molecular oxygen, can be excited to singlet oxygen ($^1O_2$), the higher energetic state of molecular oxygen, in the presence of UV light or by type II photosensitizers. Hydroperoxides are produced by the direct addition of singlet oxygen to unsaturated lipids via a non-radical pathway. Moreover, light can excite a type I photosensitizer, which acts as a photochemically activated free radical initiator. This activated sensitizer reacts with unsaturated lipids via the radical mechanism, analogous to the pathway of auto-oxidation [113].

Enzymatic oxidation includes the direct formation of hydroperoxides by lipoxygenases, and the formation of radicals by enzymes, like xanthine oxidase, which may play a role in the autoxidation process [5,111,114].

The evolution of the autoxidation process in shown in Figure 2.1. The evolution of photo- and enzymatic oxidation via the radical pathway is comparable.

An induction period can be observed in which mainly the initiation phase occurs. A rapid increase of the amount of hydroperoxides follows, which is due to the propagation phase. Since several bisallylic positions are present in an n-3 LC-PUFA chain, different hydrogen atoms can be abstracted with a low dissociation energy [115]. The large amount of different (bis)allylic positions on the n-3 LC-PUFA chain results in a complex mixture of hydroperoxides. In the case of EPA eight different hydroperoxides can be observed (hydroperoxide group on position 5, 8, 9, 11, 12, 15 and 18) and in case of DHA oxidation ten different hydroperoxides (hydroperoxide group on position 4, 7, 10, 11, 13, 14, 16, 17 and 20) [5,111] are formed. At the end, a decrease in the amount of hydroperoxides can be observed due to the termination phase and the transformation of hydroperoxides into secondary oxidation compounds [5,111].

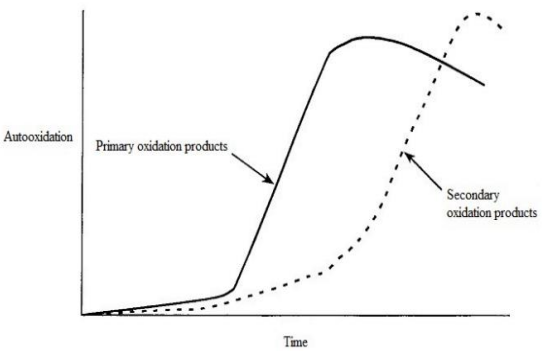


Figure 2.1. *Evolution over time of the primary and secondary autoxidation process* [5]

2.3. SECONDARY OXIDATION

The primary oxidation products are subsequently decomposed into a broad range of volatile and non-volatile secondary oxidation products [5,111]: ketones, alcohols, epoxides, aldehydes, carbohydrates, acids, but also dimers and polymers.

The formation of secondary oxidation products is also shown in Figure 2.1. Logically, this can only occur in the presence of hydroperoxides. The different hydroperoxides derived from EPA and DHA result in a complex

mixture of compounds [50]. Hammer & Schieberle [116] and Lee *et al.* [117] investigated and detected the formation of volatile compounds derived from EPA and DHA, amongst others hexanal, 2-hexenal, 2,4-heptadienal, 1-octen-3-ol, 1,5-octadien-3-one, 2,6-nonadienal, 2,4-decadienal. Different volatile compounds could be linked to an undesired fishy smell. Secondary oxidation products can further decompose or react with other compounds like proteins. The formed compounds are called tertiary oxidation products, and they explain the final decrease.

2.4. INFLUENCING FACTORS

The extent to which oxidation occurs is influenced by different environmental (oxygen, temperature, water activity and light) and intrinsic factors, like the lipid properties and the presence of endogenous antioxidants and pro-oxidants [111]. This section focusses on the most important factors related to microalgae and fruit and vegetables.

2.4.1. ENVIRONMENTAL FACTORS

Oxygen is the primary reactant in lipid oxidation reactions. The concentration, location and form of oxygen influences its reactivity. At high oxygen concentration and thus unlimited availability of oxygen, the rate of oxidation is theoretically independent of the oxygen pressure [118]. The partial oxygen pressure is only crucial when low concentrations of oxygen are present [111,115]. Removal of oxygen has thus been proven to be an efficient method to retard lipid oxidation [119]. Oxygen can be dissolved within the food matrix or non-dissolved in the headspace above the food or trapped in solid or semisolid food matrices. The proximity and accessibility of dissolved oxygen determines the oxidative stability [115]. Oxygen can be present as triplet oxygen, the lower energetic and more common state or as singlet oxygen, the more reactive form. Moreover, oxygen can be present as a reactive oxygen species, such as superoxide anion radical (O_2^-*), hydrogen peroxide (H_2O_2), hydroxyl radical (OH*). Reactive oxygen species are formed when oxygen is reduced during the

oxidation of other compounds and accelerates lipid oxidation directly as a precursor of lipid radicals or indirectly by reducing transition metals to the more active state [115].

In addition to oxygen, temperature, water activity and light can highly influence the oxidative stability. According to the Arrhenius equation, an increase of the temperature with 10°C doubles the rate of oxidation. Above 60°C other oxidation mechanisms occur due to a decrease in oxygen solubility, making that polymerization and cyclization reactions become more important [120]. A minimum lipid oxidation rate is obtained at a water activity of 0.3. Higher values lead to an increased mobility of the substrates and lower values lead to a higher accessibility of oxygen. Light is an initiator of the oxidation process and helps to accelerate the initiation phase [111].

2.4.2. LIPID PROPERTIES

The intrinsic properties of the lipids are important. The high susceptibility of n-3 LC-PUFA to lipid oxidation can be explained by the rate of the rate determining step (3). The dissociation energy needed to abstract a hydrogen decreases with an increased number of double bounds. An allylic hydrogen, a hydrogen bound to a carbon located next to a double bound carbon, is the energetically favorable position. The dissociation energy further decreases if the hydrogen is bisallylic [112]. N-3 LC-PUFA contains several bisallylic hydrogens.

Moreover, the form of the lipid seems to be an important factor in the determination of the oxidative stability. Song *et al.* [121] showed that DHA oil was more stable when incorporated in phospholipids compared to DHA incorporated in triacylglycerols. This was explained by the synergistic interaction of tocopherols and phospholipids. The exact mechanism responsible for this effect is not very well understood, but seems to be related to the involvement of phospholipids with an amino group in the regeneration of tocopherol, by hydrogen transfer [122]. Free fatty acids however are more prone to oxidation compared to their triacylglycerol form [123].

2.4.3. ANTIOXIDANTS

Antioxidants are compounds capable of preventing or retarding lipid oxidation. They can do so by scavenging radicals, quenching singlet oxygen, inactivation of reactive oxygen species, chelation of metal ions, quenching of secondary oxidation compounds or the inhibition of pro-oxidative enzymes [110]. Based on their mode of action, they are classified into primary antioxidants that break the chain reaction of oxidation by scavenging free radical intermediates, and secondary antioxidants that prevent or retard oxidation by suppression of oxidation initiators or accelerators or regeneration of primary antioxidants. The most well-known antioxidant compounds originating from microalgae and fruit and vegetables are carotenoids, phenolic compounds, tocopherols and ascorbic acid [111,124].

2.4.3.1. CAROTENOIDS

Carotenoids are polyenes built up of eight isoprene units (Figure 2.2a). They can be divided into carotenes (Figure 2.2b), which only contain hydrogen and carbon, and xanthophylls (Figure 2.2c), which contain hydrogen, carbon and minimum one oxygen [125–127]. Due to their chemical structure, carotenoids absorb light at wavelengths between 400 – 500 nm [128], resulting in a yellow, orange or red color. All photosynthetic organisms produce carotenoids because of their essential role in the photosynthesis process [129].

Carotenoids can function as antioxidants according to two different mechanisms depending on the lipid oxidation mechanism and the oxygen pressure. They are capable to capture singlet oxygen or they can scavenge peroxyl radicals by electron transfer (reaction 2.7), addition (reaction 2.8) or hydrogen abstraction (reaction 2.9) [111,130].

$$ROO^* + AH \rightarrow ROO^- + AH^{+*} \quad (2.7)$$

$$ROO^* + AH \rightarrow ROO\text{-}AH^* \quad (2.8)$$

$$ROO^* + AH \rightarrow ROOH + A^* \quad (2.9)$$

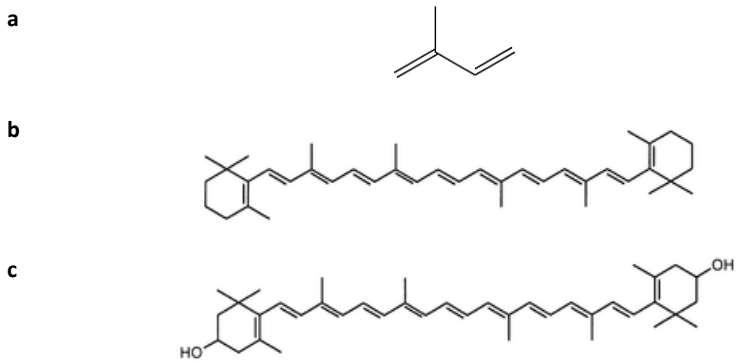


Figure 2.2. *Molecular structure of an isoprene unit (a), β-carotene as an example of a carotene (b) and zeaxanthin as an example of a xanthophyll (c)* [127]

The antioxidant capacity of carotenoids depends on their chemical structure (number of conjugated double bounds, end groups, number and position of functional groups etc.) and environmental conditions [130]. The latter include the oxygen concentration, the pH of the matrix, etc. Due to the complex antioxidant mechanism, no generalized correlation between the different factors and the antioxidant capacity can be expressed [130–132].

2.4.3.2. PHENOLIC COMPOUNDS

Phenolic compounds contain one or more aromatic rings which contain one or more hydroxyl groups (Figure 2.3) [133]. Phenolic compounds, in particular polyphenolic flavonoids, contain high antioxidant capacities by scavenging of lipid radicals (mainly by hydrogen abstraction), inactivation of reactive oxygen species and chelating metal ions and pro-oxidative enzymes [134]. The activity of phenolic compounds is influenced by the pH.

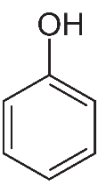


Figure 2.3. *Molecular structure of phenol* [124]

Methylated phenolic compounds, tocopherols and tocotrienols (Figure 2.4), are often considered as a separate group although they also contain an aromatic ring and a hydroxyl group. The methylated side chain of tocopherols and tocotrienols respectively contains none and three unsaturated bounds. These compounds are able to reduce peroxyl radicals and quench singlet oxygen [134,135].

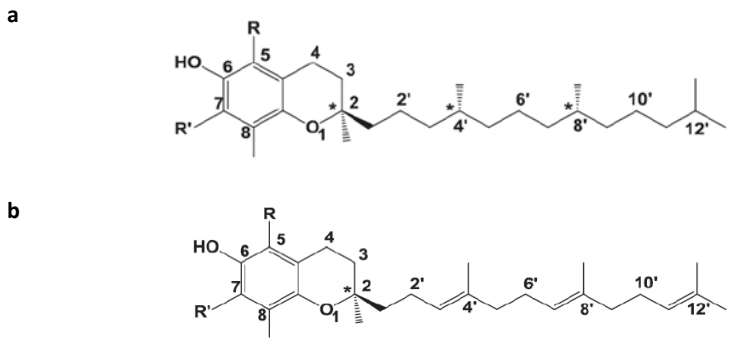


Figure 2.4. *Molecular structure of tocopherol (a) and tocotrienol (b)* [135]

2.4.3.3. ASCORBIC ACID

Ascorbic acid, vitamin C (Figure 2.5), is water soluble and exerts its antioxidant properties by scavenging free radicals (mainly by hydrogen abstraction), quenching oxygen and chelating metal ions [110,136]. By hydrogen abstraction, ascorbic acid is transformed into an ascorbate anion, which is stabilized by electron delocalization by resonance structures [114].

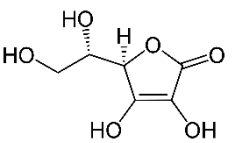


Figure 2.5. *Molecular structure of ascorbic acid* [137]

2.4.3.4. SYNERGISTIC EFFECTS

Different antioxidants show a higher antioxidant capacity when they are combined, compared to what would be expected based on their

individual capacities. The antioxidant capacity of phenolic compounds (including tocopherols) can be regenerated due to the transformation of their radical into their original state by ascorbic acid [5,138]. Carotenoids have shown synergistic effects by performing better as a carotenoid mixture compared to individual carotenoids [130].

2.4.3.5. PRO-OXIDANT EFFECT OF ANTIOXIDANTS

Under certain conditions, antioxidants can have an opposite effect. At high concentrations of the earlier mentioned antioxidants, pro-oxidative effects were observed [114]. Quantification of these concentrations is difficult as the effect is influenced by environmental factors, the chemical structure of the antioxidant and the composition of the sample [134,139].

2.4.4. PRO-OXIDANTS

Several compounds, formed hydroperoxides, hemeproteins, metal ions, chlorophyll a and free fatty acids, are labeled as pro-oxidants [111]. The role of hydroperoxides in the oxidation process is described in section 2.2 and hemeproteins are not present in microalgae or fruit and vegetables and will therefore not be discussed further in this section.

Metal ions present in the cultivation medium can be co-harvested with the microalgal biomass. Transition metals, like iron and copper, catalyze the dissociation reaction of hydroperoxides [115]. The radicals formed by this reaction (reaction 2.10 and 2.11), then participate in the autoxidation process [111,115].

$$ROOH + Me^{n+} \rightarrow RO^* + OH^- + Me^{(n+1)+} \quad (2.10)$$

$$ROOH + Me^{(n+1)+} \rightarrow ROO^* + H^+ + Me^{n+} \quad (2.11)$$

Chlorophyll a and chlorophyll a degradation products are well-known pro-oxidants during light exposed storage, although in dark conditions antioxidant effects are attributed to these compounds. However, the presence of these compounds is of inferior importance compared to carotenoids in the maintenance of the oxidative stability [140].

Free fatty acids accelerate the decomposition of hydroperoxides into free radicals by the formation of a complex between hydroperoxides and carboxyl groups through a hydrogen bound and thus exert a pro-oxidative effect [141].

2.5. LIPID OXIDATION ANALYSES

Numerous analytical methods, covering different pathways of the complex oxidation process, are available to measure lipid oxidation. They are classified in five groups based on what they measure: the absorption of oxygen, the loss of initial substrate, the formation of free radicals, primary oxidation products and secondary oxidation products [142]. The most commonly used analytical methods belong to the latter two groups. They can be divided in more simple and low-cost methods, using titration or spectrometry and more complex methods, using chromatography, Fourier-transform infrared spectrometry and nuclear magnetic resonance spectrometry [142–144]. The principles, advantages and disadvantages of these methods have been described in-depth in several reviews [5,142,145]. Therefore, only the most commonly used methods for measuring primary and secondary oxidation products are discussed in more detail in section 2.5.1 and 2.5.2.

2.5.1. PRIMARY OXIDATION

2.5.1.1. IODOMETRIC TITRATION

The iodometric titration is the standard method for the determination of the amount of hydroperoxides, expressed as the peroxide value. This method is based on the reduction of lipid hydroperoxides with potassium iodide in an acidic solution with the formation of iodine (2.12). The formed iodine is then titrated with a standardized solution of sodium thiosulfate (2.13). Starch is often used to indicate the endpoint of the titration [5,145].

$$ROOH + 2\ H^+ + 2\ KI \rightarrow I_2 + ROH + H_2O + 2\ K^+ \qquad (2.12)$$

$$I_2 + 2\ Na_2S_2O_3 \rightarrow Na_2S_4O_6 + 2\ NaI \qquad (2.13)$$

The simplicity is the biggest asset of this method, which is however time-consuming and labor-intensive. Moreover, it requires a large amount of sample and creates a significant amount of chemical waste [5,142,143]. The determination of the endpoint is arbitrary as it is based on a color change, which is difficult in colored samples. This makes the reproducibility of the method rather low.

2.5.1.2. SPECTROPHOTOMETRIC METHODS

i. *FERROUS OXIDATION METHODS*

The ferrous oxidation xylenol orange (FOX) method and the ferric thiocyanate based method of the International Dairy Federation (IDF) are based on the same principle. Hydroperoxides initiate the oxidation of ferrous ion (Fe^{2+}) to ferric ion (Fe^{3+}) in an acidic medium (2.14). Ferric ions form a blue-purple colored complex with xylenol orange (2.15), in case of the FOX method, or a red colored complex with thiocyanate (2.16), in case of the IDF method [145]. The intensity of the color formation is measured spectrophotometrically at respectively 560 and 500 nm [146,147].

$$ROOH + 2\ Fe^{2+} + H^+ \rightarrow 2\ Fe^{3+} + ROH + OH^- \qquad (2.14)$$

$$Fe^{3+} + XO \rightarrow Fe^{3+}{-}XO \qquad (2.15)$$

$$Fe^{3+} + SCN \rightarrow Fe^{3+}{-}SCN \qquad (2.16)$$

The amount of sample needed for the spectrophotometric-based methods is lower compared to the traditional titration method. As the detection is less arbitrary, the reproducibility is higher compared to the iodometric titration. Moreover, the detection limit is lower compared to iodometric titration. The largest limitation to these methods is the possible interference of compounds absorbing at the wavelength of measuring or compounds forming a complex with xylenol orange or thiocyanate [5,142].

ii. *CONJUGATED DIENES AND TRIENES (CD & CT)*

Conjugated dienes (CD) and trienes (CT), formed during the formation of hydroperoxides, can be detected based on their absorption peak at 234 and 268 nm respectively. The formation of lipid radicals leads to a shift in double bound position causing conjugated lipids (2.17) [145]. A good correlation between the amount of conjugated dienes and the peroxide value has been found [148,149].

$$-CH=CH-CH_2-CH=CH- \rightarrow$$

$$-CH=CH-CH=CH-{}^{*}CH- \text{ and } -{}^{*}CH-CH=CH-CH=CH- \quad (2.17)$$

CD & CT are the first products formed during oxidation, which implies that this method is a good indicator of the initiation of oxidation. According to other spectrophotometric methods, low amounts of sample are needed and the assay is simple. This method had the disadvantage that the absorbance maximum is difficult to determine which means that the selected wavelengths are an average. Besides, many solvents absorb at this wavelength as well [145].

2.5.2. SECONDARY OXIDATION

2.5.2.1. SPECTROPHOTOMETRIC METHODS

i. *THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS)*

The TBARS method is based on the reaction of the secondary oxidation product malondialdehyde with thiobarbituric acid (Figure 2.6). The colored complex is measured spectrophotometrically at 535 nm [5,145].

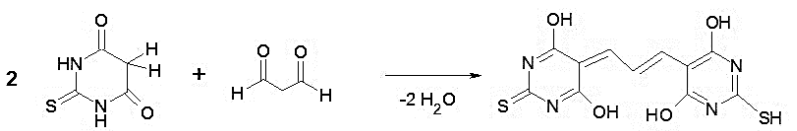


Figure 2.6. *The reaction of thiobarbituric acid with malondialdehyde* [145]

The TBARS method is controversial although it has been used extensively. Malondialdehyde, on which the method is based, is a minor secondary oxidation product. Moreover, the reaction is non-specific and

thiobarbituric acid reacts with a range of secondary oxidation compounds: alkanals, alkenals, aldehydes, etc. but also with protein amine groups, starch and sugar. Many factors affect the formation of the colored complex: temperature and time of heating, pH, metal ions, antioxidants, etc. The literature reports conflicting results of the TBARS method. Due to the low specificity and sensitivity, the method should be avoided especially in complex systems [5,145,150].

ii. *P-ANISIDINE VALUE*

The reaction of *p*-anisidine with unsaturated aldehydes to form a complex that absorbs at 350 nm according to the reaction is presented in Figure 2.7 [145].

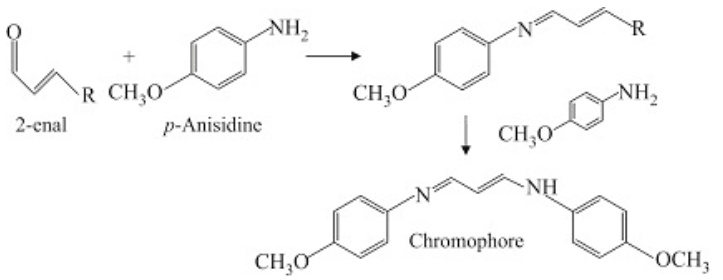


Figure 2.7. *The reaction of unsaturated aldehydes with p-anisidine* [145]

Although this method is commonly used, it has some drawbacks. *p*-anisidine can causes blood damage and is thus highly toxic. Moreover, the reactions are neither quantitative nor specific. All aldehydes react, but their response factors differ. The intensity of the formed color complex is higher for unsaturated aldehydes. Besides, *p*-anisidine reacts slowly with hydroperoxides, meaning that not only secondary oxidation products are measured. As in other spectrophotometric methods interference of compounds absorbing at the measuring wavelength cannot be excluded [145,150].

2.5.2.2. GAS CHROMATOGRAPHY

Volatile compounds, mainly aldehydes and ketones, are one of the major groups of secondary oxidation compounds and strongly correlate with the

consumer perception [116]. These compounds can be measured by gas chromatography, which is a specific and sensitive method as individual molecules at low levels can be detected [5]. Due to multitude of the secondary oxidation compounds, marker compounds are often used to be linked with the lipid oxidation pathways [151]. The information obtained via gas chromatography varies with the sampling method. Absolute quantitation is in general not possible, because of the multitude of volatile compounds and thus the difficulty to find standards. Standards are not available for all lipid oxidation compounds, thus not all external standard curves can be prepared. In case of an internal standard, the compound should not modify the lipid oxidation process and should be released completely from the matrix without intervening in the headspace equilibrium [145].

i. *STATIC HEADSPACE*

Static headspace sampling is based on the direct capture of a gas sample of a sealed vial. The gas sample is collected from the headspace above a matrix. This method has been the simplest and fastest available method for the analysis of volatile compounds. However, the limited sampling size results in a low sensitivity. The equilibrium of volatile compounds between the headspace and the matrix has a determinative impact on the results. Highly volatile compounds are favored in the gas phase, which can lead to a saturation of the headspace by these compounds, which can impede evaporation of less volatile compounds. Quantitation is mostly relative based on peak areas [5,145,152].

ii. *SOLID PHASE MICRO-EXTRACTION (SPME)*

The solid phase micro-extraction is a more complex sampling method in which compounds are concentrated by adsorption on a fiber that is inserted into a sealed vial. Depending on the fiber and adsorption time, the method can be selective for certain compounds. This method was developed to drastically improve the sensitivity and selectivity. Due to the removal of volatiles to the fiber, a new equilibrium is achieved which increases the detection of less volatile compounds. Awareness for

potential fiber competition should be taken into account when using this sampling method. Analogous to the static headspace, the quantitation is not straightforward [5,145,153].

iii. *DYNAMIC HEADSPACE*

Dynamic headspace, also known as the purge-and-trap method, is a sampling method in which volatiles are continuously removed by inert gas passing through the matrix. This means that new volatiles are released from the matrix and no equilibrium is set up anymore. The inert gas, carrying the volatiles, passes an adsorbent, which traps a range of volatile and semi-volatile compounds. The complete collection of volatiles, during a long sampling time, helps to gain sensitivity and makes it possible to detect compounds that cannot be detected by static headspace or SPME. However, the long sampling time makes this method very slow. Moreover, the transfer lines and valves increase the chance of sample contamination. Opening the sample to make the connection to the system and leaks in the transfer lines may result in a loss of volatile compounds. Absolute quantitation of volatiles is achievable by injection of an internal standard on the traps [5,145,150].

2.6. GENERAL CONCLUSION

Lipid oxidation is a complex process that creates a broad range of primary and secondary oxidation products. The extent at which oxidation occurs is influenced by many environmental and intrinsic factors.

In the past decades, several analytical techniques, with each their advantages and drawbacks, have been developed covering different parts of the oxidation pathway. To make the conclusions more reliable, at least two different methods covering each another oxidation product are recommended [154]. The methods were however initially developed to follow up lipid oxidation in colorless oils. Analysis of lipid oxidation in food products is more complex with a possible interference of color or matrix [151,155]. Therefore in chapter 3, a simple and low-cost method was

developed for colored samples. This method was then further used in chapters 4 – 7 to measure primary oxidation in complex vegetable-based products enriched with colored photoautotrophic microalgae.

PART II:

MEASURING PRIMARY LIPID OXIDATION

Chapter 3:

MEASURING PRIMARY LIPID OXIDATION IN FOOD PRODUCTS ENRICHED WITH COLORED MICROALGAE

3.1. INTRODUCTION

It is known, from the literature study in chapter 2, that lipid oxidation is a complex process that creates a broad range of primary and secondary oxidation products. Most of the analytical techniques were initially developed to monitor lipid oxidation in colorless oils (section 2.5). Food products enriched with photoautotrophic, and thus colored, microalgae may show interference with these techniques.

The aim of this chapter was to develop a simple and low-cost method to measure the primary lipid oxidation in food products enriched with colored, photoautotrophic microalgae. Therefore, the amount of primary oxidation products of an aqueous suspension enriched with photoautotrophic microalgal biomass was measured by iodometric titration, FOX, IDF and CD & CT assays. Each of these methods was applied to the lipids, which were first extracted from the suspension. During this extraction undesired extra oxidation might occur, a possibility that was also investigated in this chapter as well as oxidation during the measurement itself. Additionally, interference from ferrous and ferric ions, which could be present in the microalgal biomass, was also investigated.

3.2. MATERIALS AND METHODS

3.2.1. SAMPLE PREPARATION

3.2.1.1. N-3 LC-PUFA DOSAGE FORM

A photoautotrophic brown microalga, *Isochrysis* sp. (currently named as *T-Isochrysis* sp. [78]), was selected to screen four simple and low-cost methods to measure the primary lipid oxidation in enriched suspensions. It was obtained as freeze dried biomass from Proviron (Hemiksem, Belgium).

The heterotrophic microalga *Schizochytrium* sp., which is more prone to oxidation [50], was selected to obtain more insight in the possible occurrence of oxidation of enriched suspensions during lipid extraction and during measuring the amount of hydroperoxides. The freeze dried biomass was obtained from Mara Renewables Corporation (Dartmouth, Canada).

Commercial, and thus refined, fish oil, which was used to obtain more insight in the impact of ferrous and ferric ion addition, was obtained from Inve (Dendermonde, Belgium). Commercial fish oil was used as obtained by Inve, no suspensions enriched with fish oil were made.

3.2.1.2. PREPARATION OF ENRICHED SUSPENSIONS

An appropriate amount of freeze dried microalgal biomass (*Isochrysis* or *Schizochytrium*) to obtain a concentration of 80 mg n-3 LC-PUFA (EPA+DHA)/100 g suspension was suspended in demineralized water by mild stirring for 30 min. This concentration was used as the product can then be labelled as 'a product rich in n-3 LC-PUFA' [74]. This claim can be mentioned if 80 mg EPA + DHA is present in the product per 100 g or per 100 kcal, whichever amount is limiting. In a subsequent step the pH of the suspension was lowered to pH 4 using hydrochloric acid, considering the suspension is a model system for acidic products.

3.2.1.3. MECHANICAL TREATMENT

The suspensions were mechanically treated by high pressure homogenization (Panda 2K, Gea Niro Soavi, Parma, Italy) at 100 MPa for a single pass to physically stabilize the homogenous suspension. This processing step did not target cell wall disruption [66]. The inlet of the homogenizer was thermostated at 4°C using a cryostat (Haake, Karlsruhe, Germany) and homogenized suspensions were collected in an ice bath. Each suspension was independently mechanically treated from its duplicate.

3.2.1.4. THERMAL TREATMENT

A pasteurization process, coinciding with the thermal treatment applied to acidic products, was applied to the homogenized suspensions. The suspensions were placed in glass jars (95 mm height, 45 mm diameter) with metal lids, each containing 80 mL suspension. The pasteurization process was performed in a pilot-scale water-cascading retort (Barriquand Steriflow, Paris, France) at 90°C to obtain a process value of $^{10°C}P_{90°C}$ = 3 min. The temperature profiles in the suspensions were recorded using thermocouples and registered by the Ellab Valsuite Plus software (Ellab, Hillerød, Denmark). Each suspension was independently thermally treated from its duplicate.

3.2.1.5. STORAGE

After thermal processing, the jars from the same duplicate were pooled. The fraction needed for the accelerated storage test was chemically sterilized with 0.25% potassium sorbate. Amber screw-cap vials (20 mL) were filled with 7.5 mL treated suspension or 10 mg fish oil, hermetically sealed and stored for 12 weeks at 37°C. At this temperature the lipid oxidation process is accelerated compared to room temperature without modifications in the oxidation pathways [156]. Four different time points (week 0, 4, 8 and 12) were evaluated in terms of primary and secondary oxidation. The suspensions enriched with *Isochrysis* or *Schizochytrium* for primary oxidation were freeze dried after the storage time. All vials were stored at -80°C until analysis, to prevent further oxidation. The storage experiment was performed in duplicate.

3.2.2. ANALYSES

3.2.2.1. LIPID EXTRACTION

The total lipid fraction was extracted from the freeze dried enriched suspension with chloroform/methanol (1/1) according to Ryckebosch *et al.* [157] and determined gravimetrically. Briefly, to 100 mg freeze dried puree 4 mL methanol, 2 mL chloroform and 0.4 mL water were added. After vortexing the sample, 2 mL chloroform and 2 mL water were

additionally added, followed by vortexing and centrifugation (10 min, 750 g, room temperature). The upper phase was removed and the lower organic layer was kept in a clean tube. The remaining pellet was re-extracted in 4 mL chloroform/methanol (1/1), vortexed and centrifuged (10 min, 750 g, room temperature). The extract was pooled with the first extract. The previously described steps were repeated a second time and the solvent phases were pooled with those of the first extraction. The extract was filtered through a filter paper (Whatman n°1, Sigma Aldrich, Bornem, Belgium) with a sodium sulphate layer and the filter was washed with chloroform/methanol (1/1). The solvents were removed by rotary evaporation. For each suspension, the extraction was performed in duplicate.

For the **lipid extraction with argon flushing** the previously described method was adapted by deoxygenation of the solvents (chloroform, methanol, water and chloroform/methanol (1/1)) by flushing with argon during 30 min prior to use in the extraction.

For the **lipid extraction with BHT addition,** 4 mM of the synthetic antioxidant BHT (Sigma-Aldrich, Bornem, Belgium) was added to the extraction solvents (chloroform, methanol and chloroform/methanol (1/1)), prior to use in the extraction.

3.2.2.2. IODOMETRIC TITRATION

The iodometric titration was performed according to AOCS method Cd 8-53 [158].

3.2.2.3. FOX METHOD

The determination of the primary oxidation products using the FOX method, also named the FOX-2 method was performed according to Wrolstad *et al.* [159] with some slight modifications. In brief, 10 mg extracted lipids, obtained according to section 3.2.2.1, were dissolved in chloroform/methanol 7/3 (9.9 mL) and this solution was diluted 1/10 (0.99 mL dissolved extract + 8.91 mL chloroform/methanol 7/3). The absorbance of the diluted sample (A_{DS}) itself was first measured at

560 nm. This is necessary to correct for the absorption of the sample itself (especially for food products enriched with colored microalgae) but its necessity is often overlooked in current methodologies. Subsequently, xylenol orange (Sigma-Aldrich, Bornem, Belgium) (50 µL; 10 mM) and Fe^{2+} chloride solution (50 µL; 18 mM, acidified with HCl (1 µL; 10 M)) were added. The absorbance of the sample (A_S) was measured at 560 nm after exactly 5 min of incubation at room temperature. A blank of the Fe^{2+} chloride solution (A_B) was measured to determine the freshness of the Fe^{2+} solution and thus to prevent the use of a Fe^{2+} solution whereby Fe^{2+} is already oxidized to Fe^{3+}. A Fe^{3+} chloride standard solution (10 µg/mL) was prepared to set up the calibration curve of Fe^{3+} (zero to 3.1 µg Fe^{3+}/mL) versus absorbance (zero to 0.9). Samples with an absorbance outside this range were diluted additionally. The PV value was calculated by following equation (3.1):

$$PV = \frac{[(A_S - A_{DS} - A_B)\ x\ m_i]}{W x\ 55.84\ x\ 2} \quad (3.1)$$

Where A_S is the absorbance of the sample, A_{DS} is the absorbance of the diluted sample to correct for the absorbance of the sample itself, A_B is the absorbance of the blank, m_i is the inverse of the slope of the calibration curve, W is the mass of the sample (g), 55.84 is the atomic mass of Fe^{3+}. In the numerator of the equation, the residual absorbance (which is thus the absorbance corrected for the absorbance of the sample itself and blank) is multiplied by the inverse of the slope resulting in the corresponding amount of Fe^{3+} (mass). This value is divided by the molecular mass of iron (55.84 g/mole) and the sample mass, resulting in a value expressed as mmole Fe^{3+}/kg lipids. Based on the chemical reaction of the method, each mole lipid hydroperoxide can transform two moles Fe^{2+} into two moles Fe^{3+}. Therefore, the value is divided by two (factor 2 in the equation). This results in the final peroxide value, expressed as meq hydroperoxides/kg lipids.

3.2.2.4. ADAPTED FOX METHODS

i. *FOX METHOD WITH TRIPHENYLPHOSPHINE (TPP) ADDITION*

Nourooz-Zadeh *et al.* [160] suggested the addition of TPP during the determination of hydroperoxides to discriminate between the absorbance of the sample itself and the absorbance generated by the hydroperoxides. The FOX method of 3.2.2.3 was therefore adapted. Instead of adding 8.91 ml chloroform/methanol (7/3) to 0.99 mL of the dissolved sample as described in section 3.2.2.3, 7.91 mL chloroform/methanol (7/3) was added together with 1 mL, 10 mM TPP. The mixture was kept at room temperature for 15 min, to allow the hydroperoxides to react with TPP. This step was followed by adding xylenol orange and Fe^{2+} chloride solution as described earlier in section 3.2.2.3.

Based on equation 3.2, the corrected peroxide value can be calculated:

$$PV_{\text{without TPP}} - PV_{\text{with TPP}} = PV_{\text{corrected for interference}} \qquad (3.2)$$

With $PV_{\text{without TPP}}$, the peroxide value obtained via the standard FOX method (section 3.2.2.3) and $PV_{\text{with TPP}}$, the peroxide value obtained via the TPP adapted FOX method (section 3.2.2.4.i).

ii. *FOX METHOD WITH ARGON FLUSHING*

The FOX method (section 3.2.2.3) was adapted by deoxygenation of the solvent (chloroform/methanol (7/3)) by flushing with argon during 30 min prior to use.

iii. *FOX METHOD WITH BHT ADDITION*

BHT (4 mM) was added to the chloroform/methanol (7/3) mixture which was used in the method described in section 3.2.2.3.

3.2.2.5. IDF ASSAY

The IDF assay was performed according to Shantha and Decker [161]. The method is comparable with the FOX method described in section 3.2.2.3 except for some slight differences. Ammonium thiocyanate solution was

used instead of xylenol orange. To prepare this solution 30 g ammonium thiocyanate (Carl Roth, Karlsruhe, Germany) was weighed and water was added to a volume of 100 mL. Furthermore, the complex formation was measured at a wavelength of 500 nm.

3.2.2.6. CD & CT ASSAY

The CD & CT assay was performed according to the IUPAC method described by Abuzaytoun and Shahidi [162]. The extracted lipids (10 mg, section 3.2.2.1) were dissolved in iso-octane (10 mL) (Carl Roth, Karlsruhe, Germany) and 3 mL of this solution was brought into a quartz cuvette. The absorbance of the CD & CT was measured spectrophotometrically at 234 and 268 nm respectively. A blank of iso-octane (3 mL) was used as reference in the spectrophotometer. The amount of CD & CT was calculated based on equation 3.3:

$$\text{CD or CT} = (A \times F)/(W \times a) \qquad (3.3)$$

Where A represents the absorbance at 234 or 268 nm, F is the dilution factor, W is the mass of the sample (kg) and a is the slope of the calibration curve. Calibration curves were based on conjugated linoleic acid and conjugated linolenic acid (Larodan, Solna, Sweden), for conjugated dienes and trienes respectively.

3.2.2.7. DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Volatile secondary oxidation products were analyzed with head space solid phase micro-extraction gas chromatography coupled with mass spectrometry (HS-SPME GC-MS) according to Giri *et al.* [163] with some slight modifications. Briefly, the stored suspensions were incubated for 30 min at 60°C. A CAR/DVB/PDMS SPME fiber (Supelco, Sigma-Aldrich N.V., Bornem, Belgium) was used to extract the compounds for 35 min at 60°C, after which compounds were splitlessly injected. Injector and transfer lines were respectively kept at 250°C and 280°C. Helium was used as carrier gas and the following temperature-time program was used: 40°C (5 min), from 40 to 250°C (5°C/min), 250°C (5 min). A cross-linked methyl silicone column (HP-PONA, 50 m *x* 0.20 mm *x* 0.5 µm, Agilent

Technologies, Diegem, Belgium) was used. Mass spectra in the electron ionization (MS-EI) mode were generated at 70 eV, and recorded in the mass range from 40 to 250 amu (scan mode) for 60 min. Instrument control and data collection were performed using the GC-MSD Chemstation (G2070AA, Agilent Technologies). Compounds were identified using the Wiley 275 mass spectral library containing EI mass spectral data (John Wiley and Sons, Hewlett-Packard, Hoboken, NJ, USA). Individual volatiles were measured and a comparable volatile profile was observed in all enriched suspensions. Therefore, to keep the overview, compounds typically originating from lipid oxidation were selected as marker and the sum of these compounds was presented in the manuscript. The same conclusions could be drawn based on the sum of the compounds as well as on the individual compounds. Based on literature [117,164], the following volatile compounds originating from lipid oxidation were selected as indicator compounds: nonanal, 2-octenal, 1-octen-3-ol, 2,4-heptadienal, 3,5-octadien-2-one, 2,6-nonadienal and 2,4-decadienal. The semi quantitative concentrations of the identified volatile compounds were calculated as the area of the volatile indicator compound divided by the response factor of the external standard hexanal (spiked in an aqueous suspension). All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to be able to compare the impact of oxidation during storage. All analyses were done in duplicate.

3.2.2.8. DETERMINATION OF METALS

The determination of metals was based on the method of Ashoka *et al.* [165]. Briefly, extracted lipids (10 mg) obtained as described in section 3.2.2.1 were ashed overnight in a muffle oven at 550°C. The ash was subsequently dissolved in 10 mL 50% nitric acid. The metal concentration was measured with inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian, PTY LTD, Australia). For each of the screened metals an external calibration curve was set up. The amount of metals was expressed as µg/mg extracted lipids.

3.2.3. STATISTICAL ANALYSIS

Results were statistically evaluated by a one way analysis of variance (ANOVA). The differences were determined by a *post hoc* Tukey test. All statistical tests were performed with $\alpha = 0.05$ (JMP Pro 12.1, SAS Institute Inc.).

3.3. RESULTS AND DISCUSSION

3.3.1. COMPARISON OF FOUR LOW-COST ASSAYS

The formation of primary lipid oxidation products in an aqueous suspension enriched with a photoautotrophic microalga was followed during 12 weeks of storage of 37°C. Determination of peroxide value based on the standard iodometric titration failed as the color change was not visible due to color of the suspension. Therefore, no results of this assay could be shown. No further adaptations to this method were investigated as the visual determination of the endpoint is always difficult in colored samples and a large sample size is required. Figure 3.1 shows the amount of primary oxidation products formed over time as measured by three different spectrophotometric assays.

A comparable trend could be observed for the FOX and IDF method (Figure 3.1a), which is logic as the measurement of hydroperoxides is in both techniques based on the same principle. A significant increase over time was observed which implies the initiation of lipid oxidation during storage. On the other hand, no significant changes in the conjugated compounds were observed in the CD & CT measurements (Figure 3.1b). A high value was observed at week zero, after which the amount of conjugated compounds remained the same. This may indicate that during storage no further lipid oxidation occurred or that the peak maximum of the primary oxidation was located between two measuring points. However, both hypotheses were clearly aberrant from the conclusion based on the FOX and IDF method. To decide which of both observed trends was more likely, secondary lipid oxidation products were

measured by HS-SPME GC-MS (detailed results not shown). The volatile compounds showed a significant increase over storage time. These results confirmed the trend measured by the FOX and IDF method.

The CD & CT method did not seem suitable for colored samples. Possibly, other conjugated compounds, like carotenoids and phenols, in the lipid extract of aqueous suspensions enriched with photoautotrophic microalgae interfere with this method and possibly explain the high value of CD & CT at week zero [166].

No significant differences between the values obtained via the FOX and IDF method could be observed at none of the measured time points, which was expected as both techniques are based on a comparable mechanism. This also implies that compounds interfering with the reaction mechanism have the same impact on both analytical techniques. Measuring the absorbance of the sample itself (A_{DS}) and using it to correct the value, was an important improvement of the methodology especially for food products enriched with colored microalgae. The absorbance of the sample itself was however different for both techniques as the selected wavelength of measurement was different. The signal-to-noise ratio, calculated as the absorbance of the xylenol orange complex over the absorbance of the sample itself, was respectively ¼ and 3 for the IDF and FOX method at week zero.

This difference can be explained by the higher absorption of other compounds, for example carotenoids, at a wavelength of 500 nm (IDF method) compared to 560 nm (FOX method). Although the results were corrected for the absorbance of the sample itself, the lower signal-to-noise ratio may lead to an increase of the detection limit and a lower reproducibility. A higher relative standard deviation (although not significant, F-test, $\alpha = 0.05$) was indeed observed for the IDF (with an average of 23.3%) compared to the FOX method (with an average of 9.3%). Therefore, the FOX method was selected as the method of choice for colored suspensions enriched with photoautotrophic microalgae.

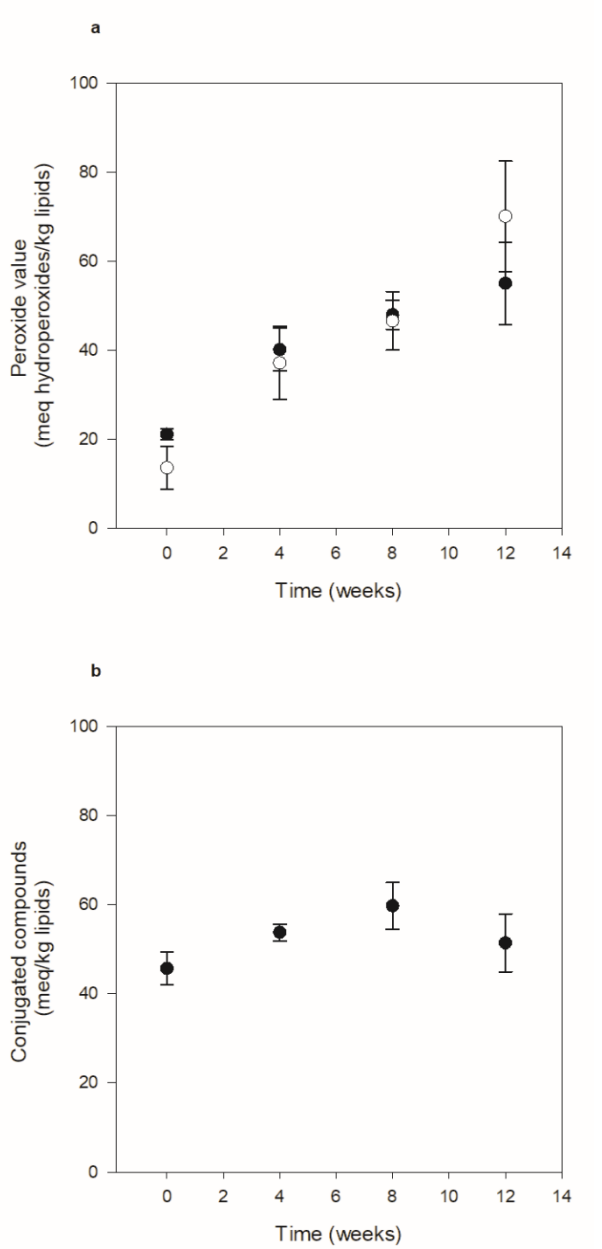


Figure 3.1. *The amount of hydroperoxides (a) or conjugated compounds (b) of an aqueous suspension enriched with photoautorophic microalgae (Isochrysis) measured by the FOX (●), IDF (○) (a) or CD & CT (●) (b) method, mean (± SD).*

3.3.2. INSIGHT IN THE FOX METHOD

In Figure 3.1a, it could be observed that even after the correction for the absorbance of the sample itself, the peroxide value at week zero was already 20 meq hydroperoxides/kg lipids. This can imply that the oxidation in the sample or during extraction already started or that other compounds interfere with the method as has been reported by Bou *et al.* [147], who suggested pigments, ascorbic acid, free iron and proteins as the main interfering compounds. Although pigments have often been suggested as interference of the FOX method [50,167], they were not a causative factor for the interference in this chapter as the peroxide value was already corrected for the absorbance of the sample itself. The FOX method was performed on a lipid extract of the enriched suspension (section 3.2.2.3), and as such it can be assumed that the more water-soluble compounds, ascorbic acid and proteins, were removed by the counter extraction with water. The presence of metal ions in the lipid extract was further investigated in section 3.3.2.1.

3.3.2.1. PRESENCE OF METAL IONS

The incorporation of microalgal biomass can imply the addition of metal ions like iron, magnesium, calcium, zinc, etc. originating from their cultivation medium. Despite that the metal ions from the cultivation medium can be co-harvested with the biomass, it is not necessarily so that these ions interfere with the FOX method as prior to this assay a lipid extraction was performed. Therefore, ICP-OES was performed on the lipid extract of the suspensions enriched with *Isochrysis*. The extracted lipids contained amounts of calcium (15.31 ± 0.03 µg/mg lipid), iron (0.084 ± 0.004 µg/mg lipid), magnesium (9.75 ± 0.03 µg/mg lipid), manganese (0.83 ± 0.03 µg/mg lipid) and zinc (0.80 ± 0.02 µg/mg lipid).

To obtain more insight in the impact of iron on the FOX method, commercial, and thus refined and free from endogenous amounts of metals, fish oil was used. Figure 3.2 shows the impact of Fe^{3+} and Fe^{2+} addition to a fresh fish oil sample.

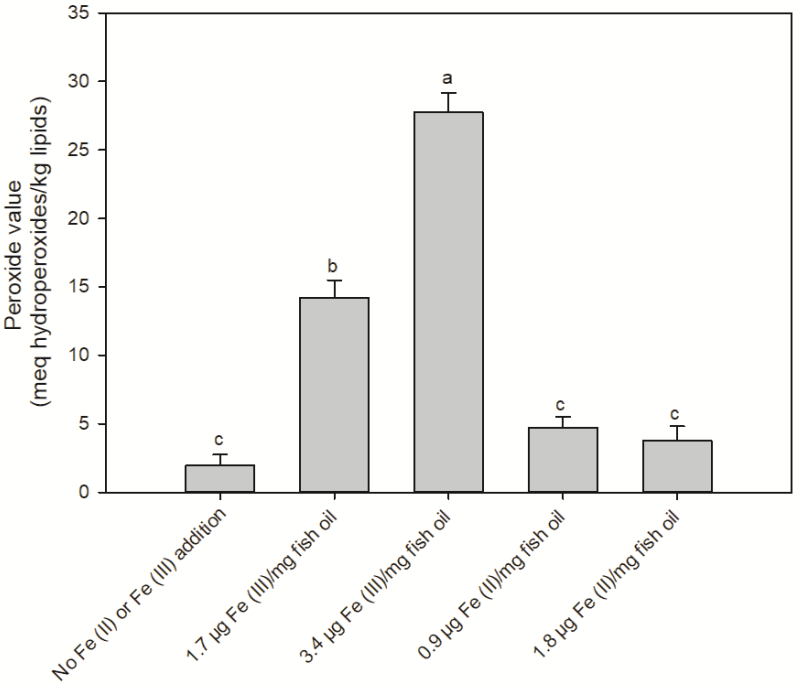


Figure 3.2. *The impact of Fe3+ and Fe2+ addition in a fresh fish oil sample on the peroxide value measured by the FOX method, expressed as meq hydroperoxides/kg lipids, mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

Addition of low amounts of Fe^{3+} (1.7 and 3.4 µg/mg fish oil respectively) resulted in a significant increase in peroxide value, which was linear with the added amount. In contrast, addition of similar amounts of Fe^{2+} (0.9 and 1.8 µg/mg fish oil respectively) had no significant impact on the peroxide value, although a slight increase could be visually observed. These results can be explained by the higher reaction rate of xylenol orange with Fe^{3+} compared to Fe^{2+} [168]. Bou *et al.* [147] suggested free iron as interfering metal, because an iron-xylenol orange complex is formed in the FOX method. This complex formation is however not selective for iron as xylenol orange also forms complexes with a large number of other metal ions depending on the pH and the ion charge density [169,170]. Amongst others Zn^{2+}, Tb^{3+}, Gd^{3+}, Cu^{2+}, Ni^{2+}, Co^{2+}, etc. have been reported as good complex formers with xylenol orange while Ca^{2+}, Mn^{2+}, etc. react weakly with xylenol orange [147,170–172]. The presence of the metal ions

observed in the lipid extract can thus possibly (partially) explain the elevated peroxide value on week zero.

3.3.2.2. REDUCTION OF INTERFERENCE

As seen in section 3.3.2.1 the presence of metal ions can interfere with the FOX method and can thus overestimate the amount of hydroperoxides. Ideally, this interference should be removed. The addition of triphenylphosphine (TPP) to the FOX method can help to eliminate the interference. TPP forms a complex with the hydroperoxides present in the sample to form TPPO [173,174]. By adding an excess of TPP, none of the hydroperoxides can oxidize Fe^{2+} to Fe^{3+}, and thus lead to an increase in the complex formation of xylenol. In the presence of TPP, all the remaining absorption is thus caused by the presence of interfering compounds like metals, etc. [160,175].

Figure 3.3 shows the impact of the addition of a supplemental step with TPP during the FOX method. The adaptation of the FOX method with TPP indeed reduced the amount of hydroperoxides measured in the sample. This suggests that the hypothesis from section 3.3.2.1 that amongst others the presence of metal ions resulted in an elevated peroxide value and thus an overestimation of the peroxide value. Analogous results were observed by using the modified FOX method with TPP on plant based materials (spinach, avocado, potato, etc.) [176]. It was however observed that both methods, with and without TPP, showed almost the same trend. Therefore, it could be concluded that the interference only had an impact on the absolute value, but not on the trend observed during storage. Besides, it was seen that the peroxide value was not reduced to zero by the adaptation of the method with TPP. This indicated that possibly the extracted oil was already oxidized, possibly during the extraction of lipids or during the FOX method itself. This was investigated in section 3.3.3.

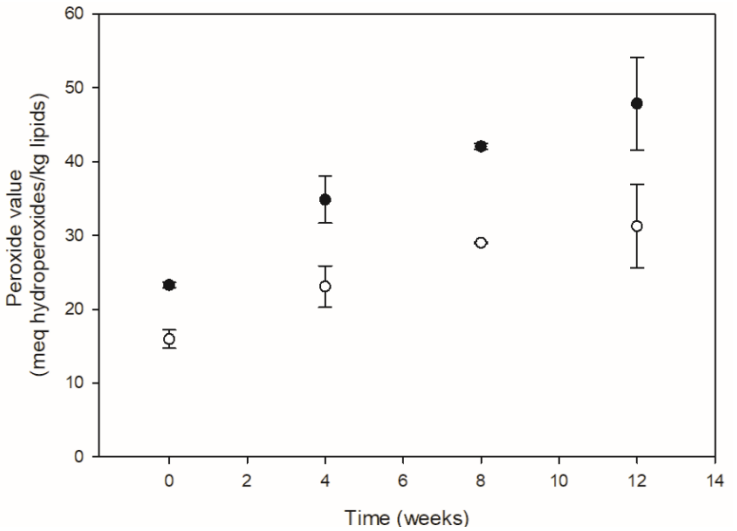


Figure 3.3. *The impact of TPP addition on the peroxide value of an aqueous suspension enriched with photoautotrophic microalgae (Isochrysis) measured by the FOX method, expressed as meq hydroperoxides/kg lipids. FOX method without TPP (●), adapted FOX method with TPP (○), mean (± SD).*

3.3.3. OXIDATION DURING ANALYSIS

As suggested in the previous section the oil could possibly be oxidized during extraction or during measuring the peroxide value. Therefore, an aqueous suspension enriched with the heterotrophic microalga *Schizochytrium* was used. This enriched suspension is more sensitive to lipid oxidation compared to the suspensions used in section 3.3.1 and 3.3.2, and therefore more suitable to study the impact of adaptations to the extraction method and analysis on lipid oxidation. After all, if in a sample prone to oxidation, no oxidation occurs during extraction or measuring the peroxide value this will also not be the case in a sample less prone to oxidation.

3.3.3.1. ARGON FLUSHING

Figure 3.4 shows the impact of argon flushing during lipid extraction and during the FOX method on the peroxide value. Argon flushing replaces dissolved oxygen from the solvents and extracted lipids. By removing

oxygen, the lipid oxidation process is delayed [145]. No significant differences in peroxide value between samples with and without argon flushing were observed. This indicated that no oxidation occurred during the extraction of the lipids, or during the FOX method and thus that the preventive action of flushing with argon has no additional value. To the best of our knowledge, no results about the impact of argon flushing on the lipid extraction and on the FOX method have been described earlier.

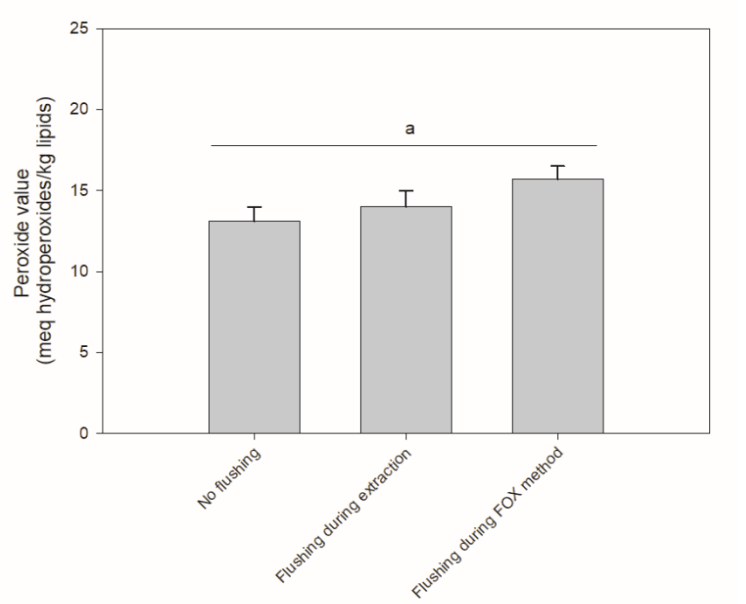


Figure 3.4. *Impact of argon flushing during extraction and FOX method on the peroxide value of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as meq hydroperoxides/kg lipids, mean (± SD). No statistical differences were observed, α=0.05.*

3.3.3.2. BHT ADDITION

BHT, a synthetic antioxidant, can inhibit lipid oxidation as a chain breaking antioxidant [177]. Figure 3.5a shows the impact of BHT addition during lipid extraction and during the FOX method on the peroxide value. It can be observed that addition of BHT during lipid extraction reduced the peroxide value significantly, while no impact of BHT addition during the FOX method was observed. More detailed analysis of the lipid extraction,

Figure 3.5b, however showed that BHT addition during extraction also significantly increased the total lipid content. The total extracted lipids increased from 72% to 102%, which is above the theoretical maximum lipid content of 100%, and much higher than the expected value for the freeze dried suspension.

This increased lipid content could be explained by a co-extraction of BHT with the lipids, implying that not all compounds in the lipid extract were indeed lipids. This was proven by a blank extraction in the presence of BHT, in which more than 95% of the BHT was co-extracted and was thus counted as 'lipids' (results not shown). This means that the amount of lipids used for the FOX method was underestimated, which explains the lower peroxide value obtained by BHT addition during extraction. A recalculation of the peroxide value based on the real amount of lipids in the extract (the obtained peroxide value divided by the following ratio: $\frac{Lipid\ content\ via\ extraction\ without\ BHT}{Lipid\ content\ via\ extraction\ with\ BHT}$) resulted in a value of 425 ± 10 meq hydroperoxides/kg lipids. From this, it could thus be concluded that the addition of BHT had no significant effect during lipid extraction. These results indicated that no oxidation occurred during the extraction of the lipids and thus that no further preventive actions were needed. No significant effect of BHT addition, and thus no oxidation, during the FOX method could be observed either, which was in line with the results of Södergren *et al.* [175]. The hypothesis, that the extracted oil was oxidized during the extraction of lipids or during the FOX method itself, suggested in section 3.3.2 could thus be rejected.

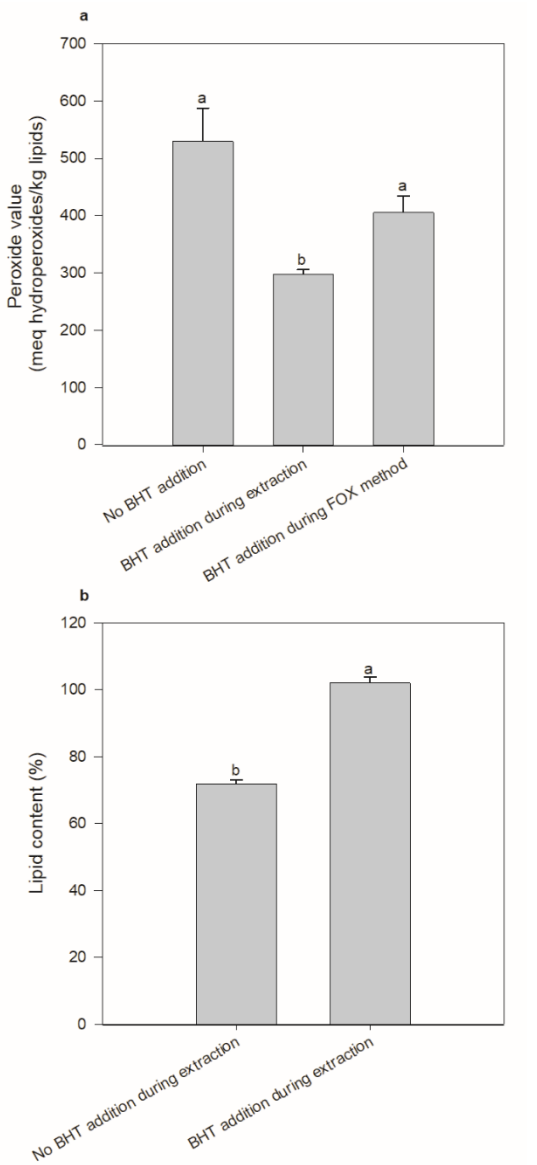


Figure 3.5. *Impact of BHT addition during extraction and FOX method on the peroxide value of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as meq hydroperoxides/kg lipids (a). Impact of BHT addition during extraction on the total lipid content of an aqueous suspension enriched with heterotrophic microalgae (Schizochytrium), expressed as % (b), mean (± SD). Statistical differences are indicated with a different letter, α=0.05. The used Schizochytrium was originating from another batch compared to the Schizochytrium used in Figure 3.4, which explains the difference in peroxide value.*

3.4. CONCLUSIONS

Different methods were screened for their potential in the determination of primary oxidation products in suspensions enriched with colored photoautotrophic microalgae. The standard iodometric titration was not valuable as the color change was not observable in the colored sample and therefore this assay could not be used. The spectrophotometric methods, FOX, IDF and CD & CT, could be used for the determination of lipid hydroperoxides (FOX and IDF) or conjugated compounds (CD & CT). In contrast to the results obtained by the FOX and IDF method, the trend obtained with the CD & CT assay was not consistent with the results of the secondary oxidation. Therefore, the CD & CT method was not selected for further optimization. Possibly the high interference of chromophore compounds with conjugated bounds has resulted in the aberrant trend. Only limited differences could be observed between the FOX and the IDF method as both methods are based on the same principle. Measuring the absorbance of the sample itself to correct the obtained value improved the methodology by excluding interference of the color of the sample. The absorbance of the sample itself was much higher for the IDF compared to the FOX method resulting in a higher (although not significant) relative standard deviation of the former. Based on this observation, the FOX method was selected as the preferred method for colored suspensions.

Despite the FOX method being selected, interference by the presence of metal ions occurred. This could partially be solved by the addition of a supplemental step in which TPP was added to eliminate the interference. The trend observed in the standard peroxide value and peroxide value corrected with TPP showed almost the same trend. Therefore, it could be concluded that the interference only had an impact on the absolute value, but not on the trend observed during storage. Furthermore it was observed that enriched suspensions sensitive to lipid oxidation did not oxidize during lipid extraction nor during the FOX method and thus this could also not explain the higher than zero values at the first time point.

Therefore, it is suggested, based on previous observations, to measure the lipid hydroperoxides in suspensions enriched with colored photoautotrophic microalgae with the FOX method and express them as delta values, which is the relative increase or decrease compared to week zero, to investigate the oxidative stability during storage. This allows to compare the trend of different samples, irrespective of the interference. This technique will therefore be applied in chapters 4 − 7 to measure primary lipid oxidation.

PART III:

POTENTIAL OF MICROALGAE IN VEGETABLE PUREES

Chapter 4:

IMPACT OF PROCESSING ON N-3 LC-PUFA IN MODEL SYSTEMS ENRICHED WITH MICROALGAE

This chapter is based on:

Gheysen, L., Bernaerts, T., Bruneel, C., Goiris, K., Van Durme, J., Van Loey, A., De Cooman, L., Foubert, I. (2018). Impact of processing on n-3 LC-PUFA in model systems enriched with microalgae. *Food Chemistry*, 268: 441–450.

4.1. INTRODUCTION

From the literature study (chapter 1), it is clear that very little research has been performed on n-3 LC-PUFA enrichment of food products by microalgae addressing processing effects and oxidative stability during storage. There are no studies focusing on the enrichment of fruit and vegetable-based products with microalgae.

Before studying the interaction with real vegetable purees (chapters 5 – 7), this chapter focused on aqueous acidic model systems enriched with microalgal biomass. The effect of mechanical (high pressure homogenization) followed by thermal (pasteurization) processing was investigated. All enriched model suspensions were characterized for their amount of n-3 LC-PUFA, free fatty acids, carotenoids and lipid polymers. Moreover, the oxidative stability of the enriched model systems was followed during a 12 week storage experiment by measuring the primary and secondary lipid oxidation products. It is hypothesized that the oxidative stability of suspensions enriched with different microalgal species may be affected by the presence of different endogenous antioxidants in different microalgae. Microalgae are unicellular organisms, including photoautotrophic and heterotrophic species. Photoautotrophic microalgae obtain their energy from photosynthesis and they contain, besides n-3 LC-PUFA, other high value compounds like antioxidants [4]. Heterotrophic microalgae grow by fermentation on organic substrates without light [13]. Although they contain a high amount of lipids (70%) they lack carotenoids [16]. Therefore, suspensions enriched with photoautotrophic microalgae are expected to be more oxidatively stable than those enriched with heterotrophic microalgae. Furthermore, it is hypothesized that processing steps may influence the amount of endogenous antioxidants and by association, the oxidative stability.

4.2. MATERIALS AND METHODS

4.2.1. MICROALGAL BIOMASS

Five different n-3 LC-PUFA rich microalgae (4 photoautotrophic and 1 heterotrophic) were used in this chapter. The biomass was obtained from different companies: *Isochrysis* sp. and *Nannochloropsis* sp., currently named as *Microchloropsis* sp. [85], (both photoautotrophic) from Proviron (Hemiksem, Belgium) and *Schizochytrium* sp. (heterotrophic) from Mara Renewables Corporation (Dartmouth, Canada) were delivered as freeze dried biomass. *Phaeodactylum* sp. and *Porphyridium* sp. (both photoautotrophic) were obtained from Necton (Olhão, Portugal) as a wet paste and were freeze dried at Proviron (Hemiksem, Belgium). All biomasses were stored at -80°C until use. The n-3 LC-PUFA content, fatty acid composition and lipid class composition of the biomass is shown in Table 4.1. The biomass was characterized in terms of total lipids, n-3 LC-PUFA, free fatty acid content and fatty acid composition.

4.2.2. EXPERIMENTAL SET-UP

Figure 4.1 shows a general overview of the experimental set-up of this chapter. For each of the different microalgae, a model suspension was made according to the steps shown in Figure 4.2. At the beginning and after all the different processing steps, the suspensions were analyzed for the amount of n-3 LC-PUFA, free fatty acids, carotenoids and lipid polymers. Additionally, the processed model suspensions were stored for 12 weeks at 37°C and analyzed for the amount of primary and secondary lipid oxidation products at week 0, 4, 8 and 12. Based on statistical optimization, a *2x2* experimental set-up was selected. For each of the microalgal species used in this chapter, the suspension was prepared in twofold and treated independently from its duplicate. Each analysis was done in twofold. This means that the obtained standard deviations do not only incorporate the analytical error but also the preparation error.

Table 4.1. *Incorporation concentration (g/100 g suspension), total lipid content (mg/g dried biomass), n-3 LC-PUFA content (mg/g dried biomass), free fatty acid content (mg/g dried biomass), fatty acid composition (%) and lipid classes (%) of Isochrysis, Nannochloropsis, Phaeodactylum, Porphyridium and Schizochytrium biomass, mean (± SD). Statistical differences (per compound) with α=0.05, are indicated with a different letter.*

	Isochrysis	***Nannochloropsis***	***Phaeodactylum***	***Porphyridium***	***Schizochytrium***
Incorporation concentration (g/100 g suspension)	4.65 (± 0.02)^{b}	2.05 (± 0.02)^{c}	2.45 (± 0.02)^{c}	6.15 (± 0.02)^{a}	0.26 (± 0.02)^{d}
Total lipid content (mg/g)	265 (± 20)^{b}	294 (± 12)^{b}	180 (± 13)^{c}	105 (± 9)^{d}	732 (± 17)^{a}
n-3 LC-PUFA (mg/g)	17.3 (± 0.5)^{c}	39.1 (± 0.3)^{b}	32.6 (± 1.6)^{b}	13.0 (± 0.3)^{c}	304 (± 27)^{a}
Free fatty acids (mg/g)	11.0 (± 0.4)^{c}	8.1 (± 0.6)^{d}	45 (± 5)^{a}	23 (± 2)^{b}	11 (± 8)^{c}
Fatty acid composition (%)					
Saturated	30 (± 2)^{b}	36 (± 2)^{b}	27.7 (± 0.9)^{b}	36 (± 2)^{b}	56 (± 2)^{a}
Mono-unsaturated	15.1 (± 0.9)^{b}	30 (± 2)^{a}	17.1 (± 0.6)^{b}	10.0 (± 0.7)^{b}	3.1 (± 0.5)^{c}
Poly-unsaturated	55 (± 2)^{a}	33.8 (± 0.7)^{c}	55 (± 2)^{a}	54 (± 3)^{a}	41 (± 2)^{b}
Lipid classes (%)					
Neutral lipids	41.4 (± 1.4)^{b}	49.9 (± 0.6)^{b}	41.4 (± 0.9)^{b}	50 (± 4)^{b}	94 (± 2)^{a}
Glycolipids	31.8 (± 0.6)^{a}	23 (± 2)^{b}	31.6 (± 0.8)^{a}	31 (± 3)^{a}	3 (± 2)^{c}
Phospholipids	26.8 (± 0.8)^{a}	27 (± 2)^{a}	27.0 (± 0.2)^{a}	19 (± 2)^{b}	3 (± 2)^{c}

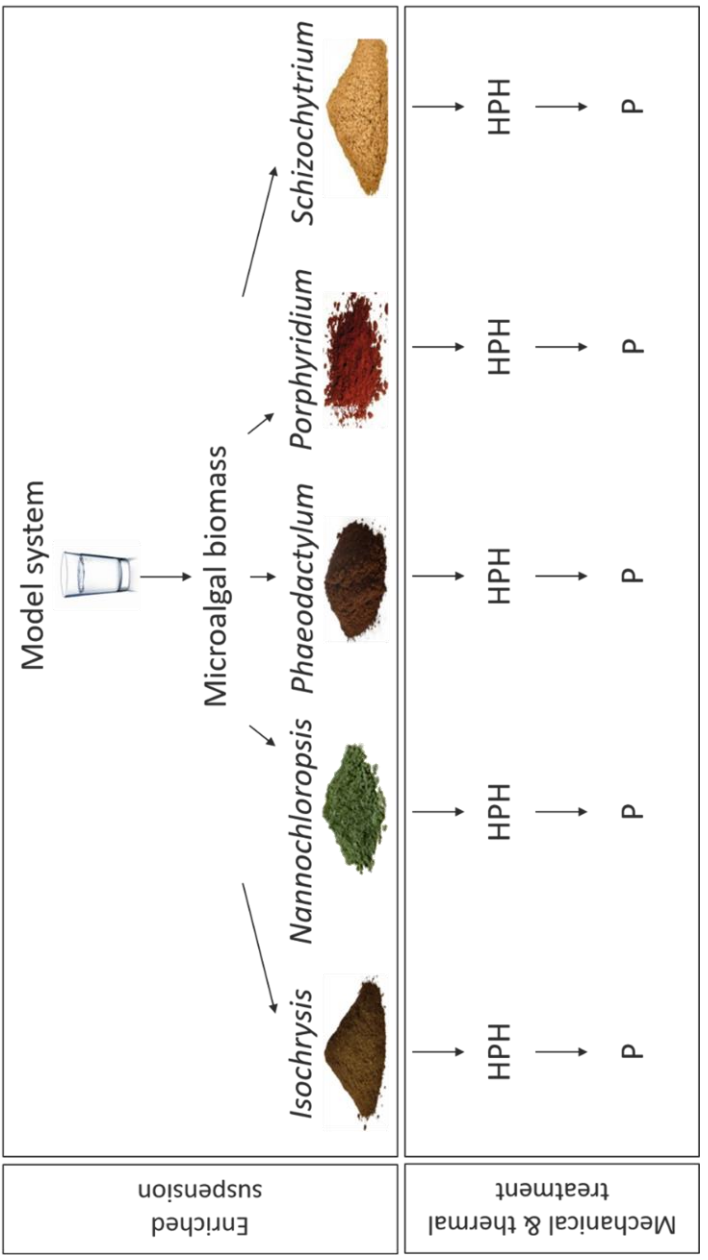


***Figure 4.1**. Experimental set-up of this chapter in which HPH stands for high pressure homogenization and P for pasteurization. Each system was made in duplicate.*

For each of the microalgae named in section 4.2.1, the enriched suspensions (with the pH lowered to 4 using hydrochloric acid) were prepared and treated according to the method described in sections 3.2.1.2-3.2.1.4.

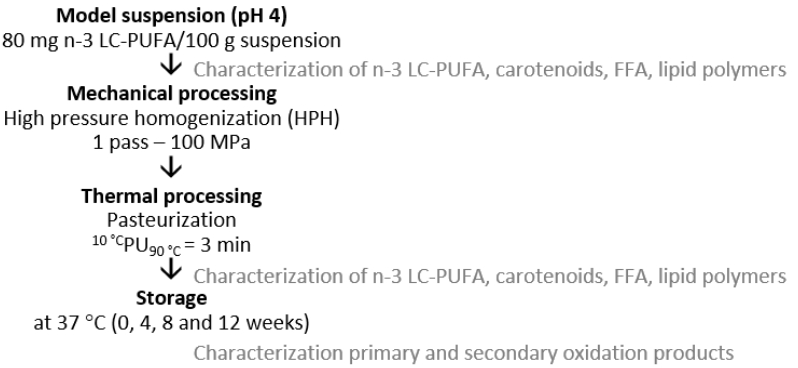


Figure 4.2. *Scheme of the different processing steps in the preparation process of enriched suspensions.*

The suspensions (except for the fraction needed in the storage experiment) were freeze dried (Christ alpha 2-4, Osterode, Germany) and stored at -80°C, until further analysis. The fraction needed for the accelerated storage test was stored according to section 3.2.1.5.

4.2.3. ANALYSES

4.2.3.1. LIPID EXTRACTION

The total lipid fraction was extracted from the freeze dried enriched suspension with chloroform/methanol (1/1) according to Ryckebosch *et al.* [157] and as described in section 3.2.2.1. The total lipid content was determined gravimetrically. An internal standard (lauric acid (C12:0)) was added for analysis of n-3 LC-PUFA and free fatty acids. For each suspension, the extraction was performed in duplicate.

4.2.3.2. DETERMINATION OF N-3 LC-PUFA CONTENT

The n-3 LC-PUFA content was determined by chromatographic separation after methylation of the extracted lipids according to Ryckebosch *et al.* [157]

with some slight modifications. Briefly, 1 mL toluene and 2 mL 1% sulfuric acid in methanol were added to 5 mg extracted lipids. This mixture was kept overnight at 50°C. After cooling down to room temperature, 5 mL 5% NaCl in water and 3 mL hexane were added. The hexane phase was diluted to a concentration of 500 ng/µL and analyzed by gas chromatography (GC) with cold on-column injection and flame ionization detection (FID) (Trace GC Ultra, Thermo Scientific, Interscience, Louvain-la-Neuve, Belgium). The following temperature-time program was applied: 70–180°C (10°C/min), 180–235°C (4°C/min), 235°C (4.75 min). The GC was equipped with an EC Wax column of length 30 m, i.d. 0.32 mm, film 0.25 µm (Grace, Lokeren, Belgium). Peak identification was performed by comparing retention times with fatty acid methyl ester (FAME) standards (Nu-Chek Prep. Inc., Elysian, USA). Peak areas were quantified based on the internal standard with Chromcard 2.5 for Windows software (Interscience, Louvain-la-Neuve, Belgium). The determination of n-3 LC-PUFA was performed in duplicate for each suspension.

4.2.3.3. DETERMINATION OF LIPID CLASSES

The separation of lipid classes was done according the method of Ryckebosch *et al.* [157]. Briefly, 20 mg of extracted lipids was dissolved in 200 µL chloroform. The lipid sample was adsorbed on a silica solid phase extraction (SPE)-column pre-rinsed with chloroform. Consecutive elution with chloroform (10 mL), acetone (10 mL) and methanol (10 mL) resulted in separation of the neutral lipids, glycolipids and phospholipids respectively. Each lipid class was determined gravimetrically. The determination of lipid classes was performed in triplicate for each microalgal biomass.

4.2.3.4. DETERMINATION OF TOTAL FREE FATTY ACID CONTENT

The free fatty acid content was determined by chromatographic separation after the formation of diethylamide derivatives according to Kangani *et al.* [178] with some slight modifications. Briefly, 10 mg extracted lipids were dissolved in 1 mL dichloromethane and 10 µL diisopropylethylamine and 30 µL diethylamine were added. The solution

was then cooled to 0°C and 40 µL bis(2-methoxyethyl) aminosulfur trifluoride was added dropwise. The sample was held at 0°C for 5 min. After warming up to room temperature, 2 mL water and 4 mL hexane were added. The solution was homogenized for 1 min and centrifuged (10 min, 750 g, room temperature). The upper organic phase (undiluted) was analyzed by the same GC-FID, as described in Section 4.2.3.2, with the following temperature-time program: 100–160°C (20°C/min), 160-240°C (4°C/min), 240°C (27 min). The determination of the free fatty acid content was performed in duplicate. Peak areas were quantified with Chromcard 2.5 for Windows software (Interscience). The peak areas were summed and compared with the area of the internal standard (C12:0) to calculate the total free fatty acid content. The determination of free fatty acids was performed in duplicate for each suspension.

4.2.3.5. DETERMINATION OF CAROTENOIDS

The carotenoid content was determined with high-performance liquid chromatography (HPLC) with photodiode array detector (PAD) according to Wright *et al.* [179]. Glass beads and 5 mL methanol were added to 25 mg of freeze dried biomass. After vortexing, the solution was centrifuged for 10 min at 750 g. The supernatant was transferred into a clean tube and the procedure was repeated 3 more times. All supernatants were pooled in the same tube. The extract was filtered (PVDF syringe filters, 4 mm, 0.2 µm) and analyzed HPLC-PAD at 436 nm (Alliance, Waters, Zellik, Belgium) with a Waters Sperisorb OD52 column (5 µm; 4.6 *x* 250 mm). The column was kept at 30°C and the samples before injection at 10°C. The mobile phases methanol/ammonium acetate buffer (80/20, pH 7.2, 0.5 M) (A), acetonitrile/ultrapure water (90/10) (B) and ethyl acetate (C) were used in following gradient program:

Time (min)	Gradient
0-4	Linear from 100% A to 100% B
4-18	Linear from 100% B to 20% B and 80% C
18-21	Linear from 20% B and 80% C to 100% B
21-24	Linear from 100% B to 100% A
24-29	Isocratic at 100% A

External calibration curves were set up for antheraxanthin, violaxanthin, diadinoxanthin, diatoxanthin, lutein, zeaxanthin, fucoxanthin (DHI, Horsholm, Denmark) and β-carotene (Sigma-Aldrich, Bornem, Belgium) to be able to express the results as µg/100 g suspension. Peak areas were quantified with Empower 2 for Windows software. The determination of carotenoids was performed in duplicate for each suspension.

4.2.3.6. DETERMINATION OF AMOUNT OF LIPID POLYMERS

The polymer content was measured according to the method of Lehtonen *et al.* [180] on the lipid samples extracted as described in 4.2.3.1. In brief, 25 mg extracted lipids were dissolved into 1 mL tetrahydrofuran (including 0.025% BHT). The dissolved samples were filtered (0.45 µm GHP, Acrodisc®; Pall Corporation, Port Washington, NY, USA) and analyzed with size-exclusion chromatography coupled with a refractive index detector (SEC-RI). The separation of compounds with a molecular weight up to 4000 g/mole was performed at 40°C on one 100-Å and two 50-Å PLGel columns (5 µm, 300 mm x 7.5mm i.d.; Polymer Laboratories Inc., MA, USA) connected in series. Tetrahydrofuran was used as mobile phase (0.6 mL/min flow). Peaks having a shorter retention time than the reference peak (27.7 min) were labeled as lipid polymers. The reference peak was rapeseed oil (comprising 98% triacylglycerols). Quantification was based on the RI response using an external standard method. Rapeseed oil triacylglycerols were used as external standard, because no suitable polymer standards were commercially available. Equal responses were assumed for the polymers as for the triacylglycerols, making the method semi-quantitative. Linear standard curves in a range of 15 µg – 30 mg/mL were set-up at the beginning and at the end of each sample sequence. All analyses were done in duplicate for each suspension.

4.2.3.7. DETERMINATION OF PRIMARY OXIDATION PRODUCTS

The determination of the primary oxidation products was performed according to the method optimized in chapter 3, described in section 3.2.2.3. All data points were expressed as delta values, which is the

relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

4.2.3.8. DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Volatile secondary oxidation products were analyzed with HS-SPME GC-MS according to section 3.2.2.7. The semi quantitative concentrations of the identified volatile compounds were calculated as the area of the volatile indicator compound divided by the response factor of the external standard hexanal (spiked in an aqueous model suspension). All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

4.2.3.9. STATISTICAL ANALYSIS

Results were statistically evaluated by a two way ANOVA model in order to assess both the impact of processing and species (for n-3 LC-PUFA, free fatty acids, carotenoids and lipid polymers) or time and species (for primary and secondary oxidation products). A multiplicative model was applied to verify whether interaction effects occurred. If not, an additive model was applied to study the effect of the variables separately. Statistical comparison of the composition of the microalgal biomass was performed by a one way ANOVA for each parameter. A *post hoc* Tukey test was used and differences were determined at a significance of $\alpha = 0.05$. All statistical analyses were performed with JMP Pro 12.1 software (SAS Institute Inc., Cary, USA).

4.3. RESULTS AND DISCUSSION

4.3.1. INFLUENCE OF PROCESSING ON N-3 LC-PUFA CONTENT

The amount of n-3 LC-PUFA in the suspension was measured before (untreated) and after the mechanical and thermal processing steps (treated). The results are shown in Figure 4.3. As for each microalgal species the initial amount of n-3 LC-PUFA in the model suspension was

standardized to a concentration of 80 mg n-3 LC-PUFA/100 g suspension, no significant differences were found between the untreated suspensions of the different species. Due to the different processing steps, a significant reduction was detected in all treated suspensions. No significant differences were seen in the remaining amount of n-3 LC-PUFA between the suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Schizochytrium.* In addition, the reduction in the amount of n-3 LC-PUFA in these suspensions was limited to a maximum of 20%. An overall recovery of more than 80% of n-3 LC-PUFA after mechanical and thermal treatment is a promising result for the use of microalgae in food applications. However, the suspensions enriched with the species *Porphyridium* showed a larger decrease in the amount of n-3 LC-PUFA compared to the suspensions enriched with the other species. In general, in all enriched suspensions a decrease in the amount of unsaturated fatty acids, mainly n-3 LC-PUFA, could be observed, while almost no change in the amount of saturated fatty acids could be detected.

The potential losses of n-3 LC-PUFA due to processing steps were described in several earlier studies. Lee *et al.* [181] reported comparable results with an overall recovery of 69% - 85% of n-3 LC-PUFA after cooking meat products enriched with heterotrophic microalgal oil. They suggested physical fat losses as an explanation for the n-3 LC-PUFA reduction. Studies on processing of products naturally rich in n-3 LC-PUFA (e.g. fish) showed similar results. Significant decreases were detected in the amount of n-3 LC-PUFA due to typical food processing steps in the studies of amongst others Cheung *et al.* [182] and Yagiz *et al.* [183]. The amount of reduction seemed to be strongly dependent on the type of food product and the used treatments. Besides the strong variations in matrix used, the explanations for the reduction in amount of n-3 LC-PUFA were very limited. Lipid oxidation was the main suggestion to explain the losses of EPA and DHA. Cheung *et al.* [182] suggested, in addition to lipid oxidation, other mechanisms such as physical losses and thermal degradation as possible causative factors for the reduction in n-3 LC-PUFA. Physical losses are typically observed by adherence to glassware, tubes of the high

pressure homogenizer, etc. As the analyses of n-3 LC-PUFA were performed on freeze dried suspensions and recalculated to the theoretically added amount of biomass per 100 g suspension, a correction for possible physical losses was already implemented. Hence, this explanation could already be eliminated as a possible causative factor. The two other suggestions will be discussed below.

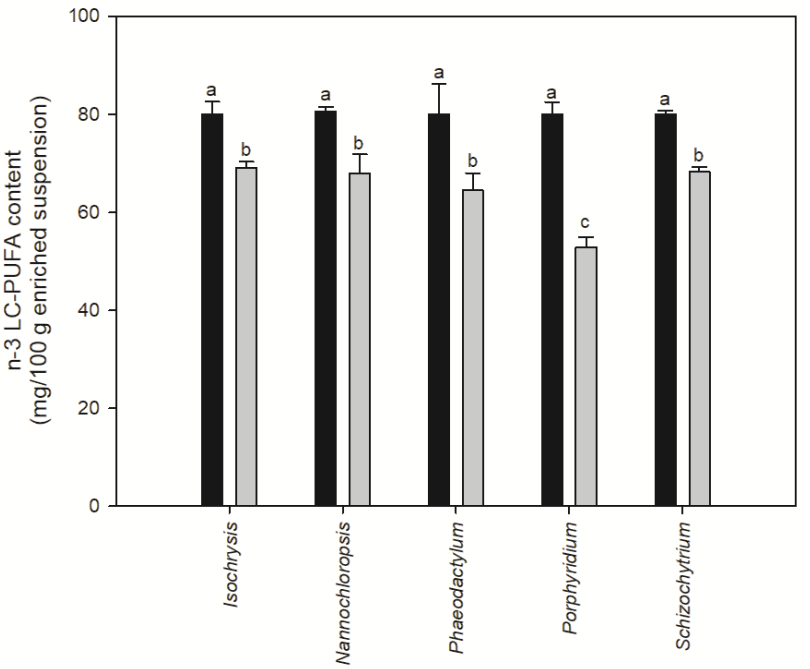


Figure 4.3. *Amount of n-3 LC-PUFA (mg/100 g enriched suspension) for suspensions enriched with Isochrysis, Nannochloropsis, Phaeodactylum, Porphyridium and Schizochytrium before (untreated, ●), and after (treated, ●) processing (high pressure homogenization +pasteurization), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

4.3.2. LIPID OXIDATION

Figure 4.4a shows the results of the primary oxidation products formed during a 12 week storage at 37°C. Clear differences in oxidative stability between the species were observed. In model suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium* a significant increase in the amount of primary oxidation products from

time point 0 to 12 weeks of storage was ascertained, which illustrates the formation of hydroperoxides. Since none of these suspensions showed a subsequent decrease in peroxide value (following the initial increase) during the 12 weeks, the oxidation process was still in the early stages. Moreover, no significant differences were detected between the suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium*. On the other hand, in the model suspensions enriched with *Schizochytrium* a significant decrease in the peroxide value from 0 to 12 weeks was observed. It is remarkable that no increase in peroxide value for these suspensions was detected. This suggests that in these suspensions a high amount of hydroperoxides (absolute values at week zero of 0.208 ± 0.015 meq hydroperoxides/100 g suspension for suspensions enriched with *Schizochytrium* compared to 0.024 ± 0.001 for suspensions enriched with photoautotrophic microalgae) was already formed during processing. Those hydroperoxides transformed into secondary oxidation products, coupled with a decrease in peroxide value, during storage. This indicates a lower oxidative stability for *Schizochytrium* compared to the other species.

The results of the secondary oxidation confirmed the results of the primary oxidation. In Figure 4.4b, again two trends could be established. Suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium* showed a small, but significant, increase in the volatiles originating from lipid oxidation. The slow increase in secondary oxidation compounds suggests again, in line with the results of the primary oxidation, that the oxidation process was still at an early stage. Again, no differences were detected between the suspensions enriched with the different microalgal species.

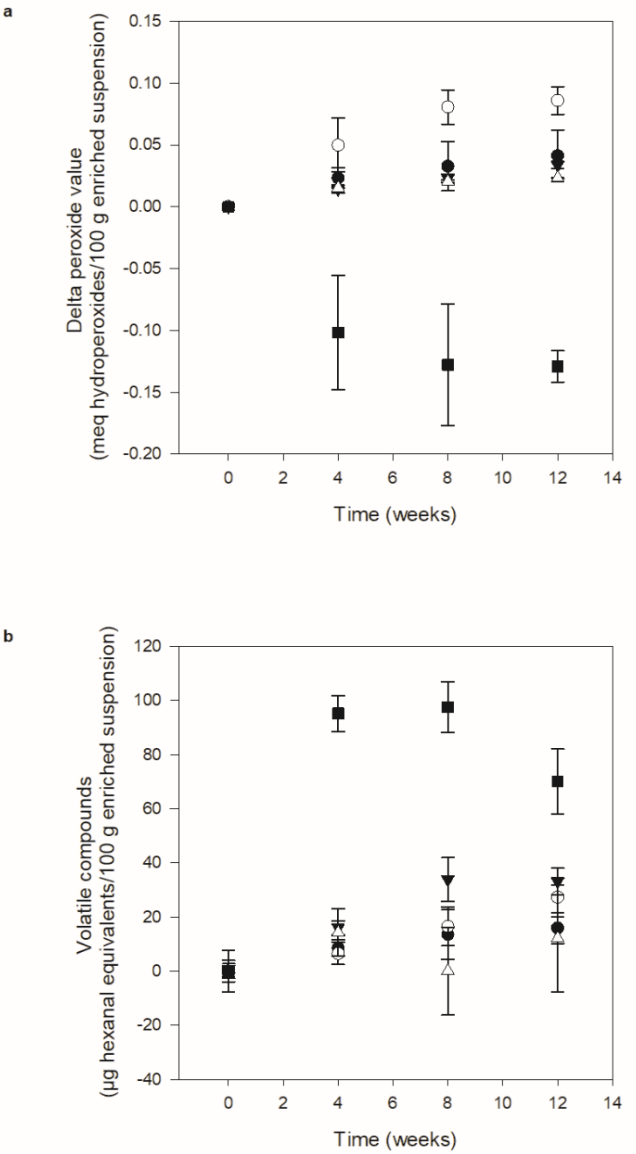


Figure 4.4. *Evolution of primary oxidation (a) and secondary oxidation (b) during 12 weeks of storage at 37°C for the suspensions enriched with Isochrysis (●), Nannochloropsis (○), Phaeodactylum (▼), Porphyridium (Δ) and Schizochytrium (■) after high pressure homogenization and pasteurization. The results are expressed as delta values, which is the relative increase or decrease compared to week zero. The peroxide value is expressed as meq hydroperoxides/100 g suspension (a) and the volatile compounds originating from lipid oxidation are expressed as mg hexanal equivalents/100 g enriched suspension (b), mean (± SD).*

Suspensions enriched with *Schizochytrium* showed a steep increase in the volatile compounds followed by a stagnation phase and even a slow decrease at the end of the storage experiment. Since primary oxidation products transform into secondary oxidation products, there is a delay between both oxidation markers [5]. This explains the difference between the tendency of the primary and secondary oxidation compounds. However, both parameters illustrated a lower oxidative stability for the model suspension enriched with *Schizochytrium*.

In conclusion, large differences could thus be observed in the oxidative stability of the enriched suspensions depending on the used microalgal species. Suspensions enriched with photoautotrophic microalgae (*Isochrysis, Nannochloropsis, Phaeodactylum* or *Porphyridium*) showed a significantly higher oxidative stability compared to suspensions enriched with heterotrophic microalgae (*Schizochytrium*).

The higher oxidative stability of model suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium* compared to suspensions enriched with *Schizochytrium* could most probably be explained by the dissimilarity in carotenoid content (Table 4.2). Large initial differences in the quantity and variety of carotenoids between the species were observed and could be explained by their growth conditions. The carotenoid containing microalgae *Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium* are photoautotrophic microalgae. This means that their growth is based on photosynthesis for which photosynthetic pigments like carotenoids are needed [184]. The fact that no carotenoids were detected in the suspensions enriched with *Schizochytrium* can thus be explained by their heterotrophic growth [13]. Suspensions enriched with *Isochrysis* and *Phaeodactylum* mainly contained fucoxanthin, suspensions enriched with *Nannochloropsis* showed a combination of mainly antheraxanthin, violaxanthin and zeaxanthin, suspensions enriched with *Porphyridium* contained mainly zeaxanthin. These results were comparable with the literature [79,185].

Table 4.2 *Carotenoids composition (mg/100 g enriched suspension) for suspensions enriched with Isochrysis, Nannochloropsis, Phaeodactylum, Porphyridium and Schizochytrium before (untreated) and after (treated) processing (high pressure homogenization+pasteurization), mean (± SD). Statistical differences (per carotenoid) are indicated with a different letter, α=0.05.*

	Isochrysis		***Nannochloropsis***		***Phaeodactylum***		***Porphyridium***		***Schizochytrium***	
	Untreated	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**	**Untreated**	**Treated**
Carotenoids										
Fucoxanthin	50.9 (± 0.3)[a]	50 (± 2)[a]	-	-	37.5 (± 0.7)[b]	28 (± 2)[c]	2.48 (± 0.14)[d]	2.3 (± 0.2)[d]	-	-
Violaxanthin	-	-	3.5 (± 0.4)[a]	0.54 (± 0.04)[b]	-	-	0.77 (± 0.07)[c]	-	-	-
Diadinoxanthin	4.20 (± 0.04)[a]	-	-	-	1.02 (±0.02)[b]	-	0.42 (± 0.07)[c]	-	-	-
Antheraxanthin	0.23 (± 0.02)[c]	-	2.1 (± 0.3)[a]	1.47 (± 0.11)[b]	-	-	-	-	-	-
Diatoxanthin	4.50 (± 0.06)[a]	3.88 (± 0.19)[b]	-	-	1.47 (± 0.02)[c]	-	-	-	-	-
Lutein	-	-	-	-	-	-	0.82 (± 0.15)[a]	0.65 (± 0.11)[a]	-	-
Zeaxanthin	-	-	1.93 (± 0.03)[c]	1.65 (± 0.13)[c]	-	-	10.8 (± 0.2)[a]	8.3 (± 1.0)[b]	-	-
β-carotene	3.02 (± 0.04)[b]	NQ	-	-	2.34 (± 0.19)[c]	NQ	5.27 (± 0.18)[a]	NQ	-	-

NQ: Not quantifiable due to overlap with chlorophyll degradation products

A reduction in the amount of carotenoids in the treated suspensions was detected in all cases. Indeed, carotenoids are sensitive to processing conditions, mainly thermal treatments [186,187]. However, some of them showed higher resistance to the treatments. In this chapter a higher stability of fucoxanthin, lutein and zeaxanthin compared to antheraxanthin, diadinoxanthin, diatoxanthin and violaxanthin was observed, which is in line with the results of Lee & Coates [186] and Mok *et al.* [188].

Carotenoids may have antioxidant activity due to their radical scavenging properties [189], with different carotenoids showing a different antioxidant capacity [130]. However, until now, it is not clear what the impact is of the type of carotenoids and their mutual cooperation on the antioxidant capacity. The lipid oxidation results suggest that the presence of carotenoids in general is more important than the specific type and amount.

The presence of carotenoids, in the photoautotrophic microalgae, probably leads to a retardation in lipid oxidation and thus a higher oxidative stability. It has been shown that n-3 LC-PUFA associated with glyco- and phospholipids are less susceptible to lipid oxidation than when associated to the neutral lipid class [190]. Thus, the lipid class composition may have also had an influence on the oxidative stability. In *Schizochytrium* biomass 94% of the lipids were neutral lipids while for autotrophic microalgae only 41-50% of the lipids were neutral lipids (see Table 4.1). Moreover Ryckebosch *et al.* [79] observed that in photoautotrophic microalgae more than 50% of the EPA and DHA was incorporated in the glyco- and phospholipids.

However, the detected amount of lipid oxidation was too limited to explain the reduction in n-3 LC-PUFA (see 4.3.1). Moreover, the highest reduction in n-3 LC-PUFA was detected in the suspensions enriched with *Porphyridium*, while the highest degree of lipid oxidation was detected in the suspensions enriched with *Schizochytrium*. As such, it can be concluded that lipid oxidation is not sufficient to explain the observed reduction of n-3 LC-PUFA.

4.3.3. THERMAL DEGRADATION OF N-3 LC-PUFA

Thermal degradation could be another causative factor for the decrease in n-3 LC-PUFA. Thermal alterations can be caused by non-oxidative as well as oxidative reactions. Studies focusing on the effect of heat under strictly non-oxidative conditions showed among others the formation of free fatty acids, cyclic monomeric triacylglycerols, isomeric monomeric triacylglycerols and non-polar di- and oligomeric triacylglycerols. Heat combined with a flow of oxygen might speed up the oxidation reaction and can result in the formation of volatile compounds and oxidized mono-, di- and oligomeric triacylglycerols [191]. In this chapter, small amounts of oxygen were present in the headspace during thermal treatment. It can therefore be assumed that both non-oxidative and oxidative reactions occurred simultaneously. Focusing only on the oxidation process was insufficient to explain the differences in the reduction of n-3 LC-PUFA between the different microalgal species (see 4.3.2). Therefore, lipid polymers were measured by SEC-RI. No particular effort was made to distinguish between non-oxidative and oxidative thermal degradation products in this regard.

The amount of lipid polymers is presented in Figure 4.5. A significant increase in amount of polymers was detected in the model systems enriched with *Porphyridium*, while no significant increase was observed in model systems enriched with *Isochrysis, Nannochloropsis* and *Schizochytrium*. Results for polymerization of model systems enriched with *Phaeodactylum* are unfortunately not available. The amount of polymerization was of the order of magnitude of the reduction in n-3 LC-PUFA. This gives an indication that thermal alterations, caused by non-oxidative as well as oxidative reactions, like polymerization could be an explanation for the decrease in the amount of n-3 LC-PUFA. Fournier *et al.* [192] showed a decrease of 90% in n-3 LC-PUFA during the deodorization of fish oil at 180, 220 and 250°C and indicated that this involved the formation of lipid polymers. Studies describing the formation of polymers in n-3 LC-PUFA rich food products at lower temperatures do not exist.

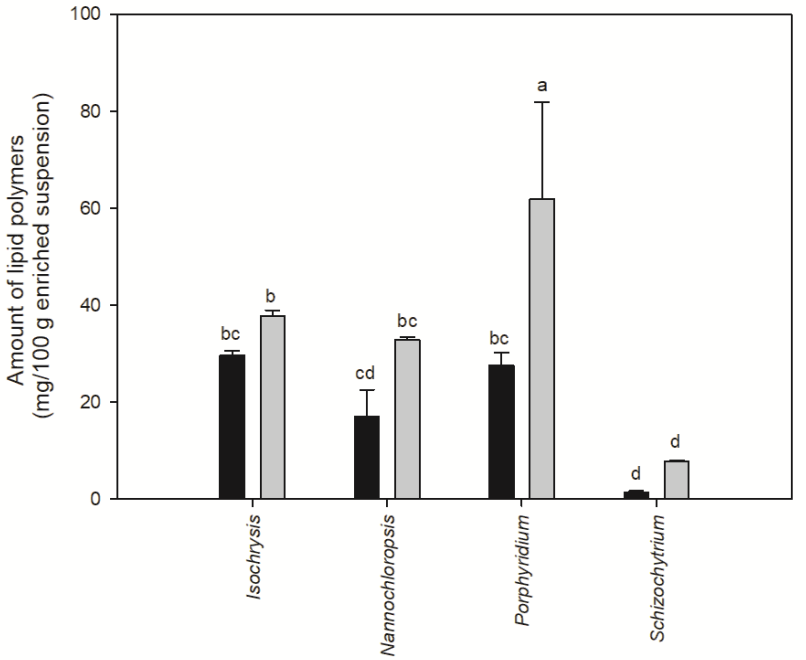


Figure 4.5. *Amount of lipid polymers (mg/100 g enriched suspension) for suspensions enriched with Isochrysis, Nannochloropsis, Porphyridium and Schizochytrium before (untreated, ●), and after (treated, ●) processing (high pressure homogenization + pasteurization), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

The difference observed in the amount of polymerization might be explained by the differences in the free fatty acid content. The untreated suspensions showed large initial differences in the amount of total free fatty acids (Figure 4.6). *Porphyridium* enriched suspensions already showed a significantly higher amount of total free fatty acids before processing than the suspensions enriched with *Isochrysis, Nannochloropsis* and *Schizochytrium*. Furthermore, a significant increase in the amount of free fatty acids due to processing was detected in suspensions enriched with *Isochrysis* and *Porphyridium*. The lowest amount of total free fatty acids after processing (4.3 mg/100 g suspension) was observed in suspensions enriched with *Schizochytrium*. Intermediate amounts of total free fatty acids were observed in treated suspensions enriched with *Isochrysis, Nannochloropsis* and

Phaeodactylum. When comparing Figures 4.5 and 4.6, it appears that the suspensions with a lower amount of total free fatty acids, showed a lower increase in polymer content. Also, there is literature evidence that EPA and DHA in the free fatty acid form are more prone to polymerization reactions than their corresponding ethyl ester form [193]. Once the fatty acids are esterified, no differences are expected between the different ester forms. To confirm this hypothesis a 'good quality' *Porphyridium* with a low initial amount of free fatty acids was processed under the same conditions. Instead of the high reduction in n-3 LC-PUFA as observed with the high free fatty acid containing *Porphyridium*, only a limited reduction (of 8%) in n-3 LC-PUFA content could be detected (detailed results not shown), confirming the hypothesis that loss of n-3 LC-PUFA is related to high amounts of free fatty acids.

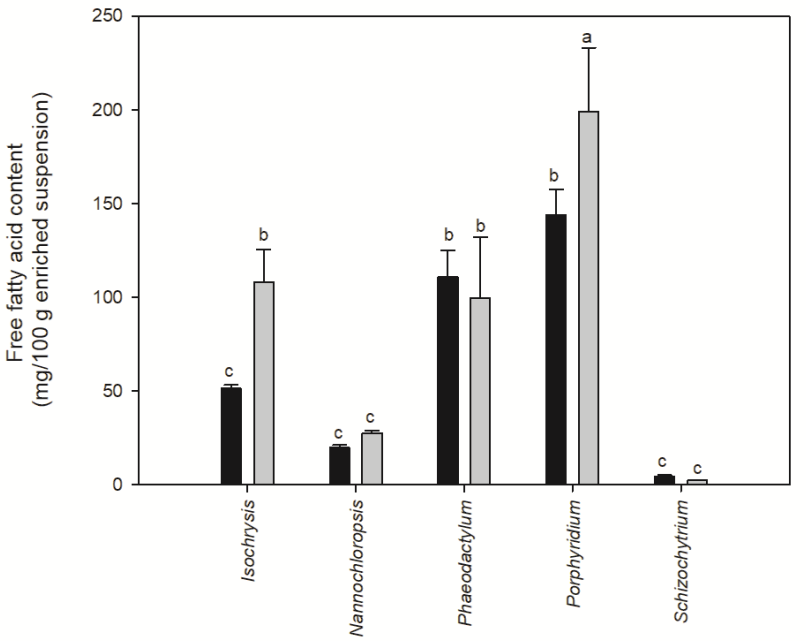


Figure 4.6. *Free fatty acid content (mg/100 g enriched suspension) for suspensions enriched with Isochrysis, Nannochloropsis, Phaeodactylum, Porphyridium and Schizochytrium before (untreated, ●), and after (treated, ●) processing (high pressure homogenization +pasteurization), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

However, due to the limitations of the current analytical techniques for complex lipid systems, conclusions based on these results of polymerization are indicative. Traditional analytical techniques allowed us to separate the lipid fractions only based on the differences in molecular weight compared to a reference. However, the complex lipid system of microalgae consisting of a neutral, glyco- and phospholipid fraction makes the separation based on size more complicated. Therefore, the hypothesis of thermal degradation and the relation with the presence of free fatty acids as the causative factor for the reduction in n-3 LC-PUFA should be investigated more in detail. However, in order to do so, more advanced analytical techniques for complex lipid systems should be developed.

4.4. CONCLUSIONS

Mechanical and thermal processing reduced the amount of n-3 LC-PUFA in model systems enriched with microalgal biomass. This reduction was however less than 20% for the model systems enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Schizochytrium*, but was more pronounced for systems enriched with *Porphyridium* with a high free fatty acid content. The reduction in n-3 LC-PUFA could not be explained by lipid oxidation. Yet, clear differences in oxidative stability between model systems enriched with photoautotrophic (*Isochrysis, Nannochloropsis, Phaeodactylum* and *Porphyridium*) and heterotrophic (*Schizochytrium*) biomass were observed. The presence of carotenoids in the photoautotrophic microalgae probably leads to a retardation in lipid oxidation and thus a higher oxidative stability. This observation supported our hypothesis that the oxidative stability of the microalgal species may be affected by the presence of endogenous antioxidants. Thermal degradation, possibly combined with extended oxidation in the case of heterotrophic microalgae, was the causative factor for the decrease in n-3 LC-PUFA. Thermal alterations, caused by non-oxidative as well as oxidative reactions, were (indicatively) detected in all treated

model systems. Especially in the *Porphyridium* system, which showed the most significant decrease in n-3 LC-PUFA, a pronounced increase in polymer content was also observed. The free fatty acid content can be related to the degree of lipid polymer formation, since it was shown that the use of *Porphyridium* with a lower initial free fatty acid content resulted in a lower reduction of n-3 LC-PUFA. From this chapter, it can thus be concluded that photoautotrophic microalgae with a low free fatty acid content are a promising source of n-3 LC-PUFA in thermally processed food systems.

Chapter 5:

IMPACT OF *NANNOCHLOROPSIS* SP. DOSAGE FORM ON THE OXIDATIVE STABILITY OF N-3 LC-PUFA ENRICHED TOMATO PUREES

This chapter is based on:

Gheysen, L., Lagae, N., Devaere, J., Goiris, K., Goos, P., Bernaerts, T., Van Loey, A., De Cooman, L., Foubert, I. (2019). Impact of *Nannochloropsis* sp. dosage form on the oxidative stability of n-3 LC-PUFA enriched tomato purees. *Food Chemistry*, 279: 389–400.

5.1. INTRODUCTION

In the previous chapter, photoautotrophic microalgae with a low free fatty acid content were shown to be a promising source of n-3 LC-PUFA in thermally processed acidic model systems. In this chapter, the transition is made from model systems to tomato puree.

From the literature study in chapter 1 it is clear that microalgae can be incorporated into the food chain in different ways: direct incorporation of the whole biomass, direct incorporation of the oil extracted from the biomass and indirect use (microalgal oil or biomass) as an animal feed supplement to enrich animal derived products. In previous research, heterotrophic microalgae were always incorporated in food products as oil, while photoautotrophic microalgae were incorporated as intact biomass. Different dosage forms of the same microalga, all resulting in an increased n-3 LC-PUFA content, may each have their own advantages and disadvantages. The use of whole biomass also implies the incorporation of endogenous antioxidants present in the microalgal biomass [185]. This may help to increase the oxidative stability of the n-3 LC-PUFA [156]. The whole biomass can be added as intact biomass or the cells can be (partially) disrupted (e.g. by high pressure homogenization) to promote the liberation of n-3 LC-PUFA and therewith increase the bioaccessibility [194]. However, a lower integrity of the cell may also result in a reduced oxidative stability. The economic impact of the supplementary disruption unit operation should be taken into consideration as well [195]. Oil extracted from the biomass is another dosage form of n-3 LC-PUFA derived from microalgae. This may lead to a more straightforward procedure for acquiring novel food status compared to the whole biomass, as some compounds are removed during extraction. On the other hand, the endogenous carotenoids are partially lost by the extraction [196] and the n-3 LC-PUFA present in the oil is not encapsulated anymore. It is hypothesized that this may lead to reduced oxidative stability. Additionally, an expensive extraction step is required to obtain this dosage form [70]. To the best of our knowledge, no studies have focused

on the impact of dosage form of n-3 LC-PUFA rich microalgae on the oxidative stability of enriched food products.

The aim of this chapter was thus to investigate in-depth the potential of photoautotrophic microalgae as an alternative source of n-3 LC-PUFA in real vegetable-based products, and especially study the impact of different microalgal dosage forms. Three dosage forms of the microalga *Nannochloropsis sp.* (intact biomass, disrupted biomass and oil) were added to a tomato puree. *Nannochloropsis* sp. was selected because of its potential shown in chapter 4 and the presence of a tough cell (wall) caused by the presence of the aliphatic non-hydrolysable biopolymer algaenan [66]. The latter offers the possibility of creating a clear difference between intact and disrupted biomass. The effect of the different microalgal dosage forms, added in an amount to reach a standardized concentration of n-3 LC-PUFA, was investigated on the amount of n-3 LC-PUFA present and on the oxidative stability in the enriched tomato puree. The impact of the dosage forms was also compared to that of fish oil, as the main commercial source of n-3 LC-PUFA. Furthermore, the impact of processing intensity was studied for the different dosage forms as well.

5.2. MATERIALS AND METHODS

5.2.1. PREPARATION OF DIFFERENT DOSAGE FORMS OF *NANNOCHLOROPSIS*

Nannochloropsis sp. was obtained from Proviron (Hemiksem, Belgium) as freeze dried biomass. The batch was split up in three parts to obtain the different dosage forms originating from the same batch. A first part was kept as received and labelled as '**intact biomass'**. A second part (named '**disrupted biomass**') was resuspended in water to obtain a 10% microalgal paste, which was (partially) disrupted by high pressure homogenization (4 passes at 100 MPa) using a Panda 2K Gea Niro Soavi (Parma, Italy) and then freeze dried. High pressure homogenization at this intensity leads to the (partial) disruption of the *Nannochloropsis* cell (wall)

(Balduyck *et al.* [66]). The third part of the biomass was used to extract the **oil** via a large scale hexane/isopropanol (3/2) extraction. This solvent mixture was selected as a food grade solvent mixture with the highest extraction efficiency according to Ryckebosch *et al.* [196]. The extraction solvent hexane/isopropanol (3/2) (3 L) was added to intact *Nannochloropsis* biomass (200 g) and stirred for 90 min. The extract was separated by vacuum filtration and the residual biomass was extracted again according to the same procedure. Both extracts were pooled and the solvent was finally removed by rotary evaporation. The oil obtained was flushed with nitrogen.

Commercial fish oil containing added antioxidants (BHA and tocopherols) was obtained from Inve (Dendermonde, Belgium).

All samples were stored at -80°C until use.

Table 5.1 shows the composition of the different n-3 LC-PUFA sources, as well as the used incorporation concentration. In this chapter, varying incorporation concentrations of the n-3 LC-PUFA sources were used in order to obtain a fixed concentration of 80 mg n-3 LC-PUFA/100 g of puree in each of the systems. At this concentration the product can be labelled as 'a product rich in omega-3' [74]. This standardized amount of n-3 LC-PUFA allows us to compare the oxidative stability of the enriched purees and asses the suitability of the dosage forms applied.

5.2.2. PREPARATION OF TOMATO PUREE

Tomatoes (cultivar *Bonaparte*) were obtained from Proefcentrum Hoogstraten (Hoogstraten, Belgium). They were cut into slices and blanched (8 min at 95°C) to inactivate endogenous enzymes. The blanched tomatoes were then blended (Büchi mixer B-400, Flawil, Switzerland) and sieved (pore size 1 mm) to remove the larger parts, seeds and fibers. The obtained tomato puree was homogenized and stored at -40°C until further use.

Table 5.1. *Composition of the different n-3 LC-PUFA sources in terms of n-3 LC-PUFA, free fatty acids and antioxidant profile, expressed as mg/g n-3 LC-PUFA source, mean (± SD). Statistical differences (per compound) with α=0.05, are indicated with a different letter.*

	Intact *Nannochloropsis*	**Disrupted *Nannochloropsis***	***Nannochloropsis* oil**	**Fish oil**
Incorporation concentration (g/100 g puree)	1.88 (± 0.01)^{b}	2.12 (± 0.01)^{a}	0.61 (± 0.01)^{c}	0.49 (± 0.01)^{d}
n-3 LC-PUFA (mg/g)	42.4 (± 0.6)^{c}	37.7 (± 1.1)^{c}	131 (± 2)^{b}	162.3 (± 1.4)^{a}
Free fatty acids (mg/g)	12.8 (± 1.4)^{c}	27.8 (± 0.9)^{b}	72 (± 5)^{a}	9.1 (± 0.7)^{c}
Carotenoids (mg/g)				
Antheraxanthin	0.85 (± 0.15)^{b}	0.28 (± 0.01)^{b}	2.37 (± 0.6)^{a}	-
Zeaxanthin	1.88 (± 0.16)^{a}	1.40 (± 0.03)^{a}	2.21 (± 0.5)^{a}	-
β-carotene	1.73 (± 0.19)^{a}	1.2 (± 0.2)^{a}	2.42 (± 0.78)^{a}	-
Phenolic compounds (mg/g)	3.3 (± 0.1)^{b}	3.1 (± 0.1)^{b}	32 (± 2)^{a}	0.87 (± 0.10)^{b}
Ascorbic Acid (mg/g)	1.21 (± 0.10)^{a}	0.42 (± 0.03)^{b}	0.06 (± 0.01)^{c}	0.06 (± 0.01)^{c}
Tocopherols (mg/g)	0.30 (± 0.01)^{b}	0.04 (± 0.01)^{c}	1.62 (± 0.01)^{a}	1.68 (± 0.03)^{a}

5.2.3. EXPERIMENTAL SET-UP

Figure 5.1 shows a general overview of the experimental set-up of this chapter. For each of the different n-3 LC-PUFA sources, a tomato puree was prepared according to the steps shown in Figure 5.2 and described more in detail in section 5.2.4. After each processing step (mixing, high pressure homogenization, pasteurization or sterilization), the purees were analyzed for the amount of n-3 LC-PUFA, free fatty acids, carotenoids, tocopherols, phenolic compounds and ascorbic acid. Moreover, the enriched tomato purees were stored for 12 weeks at 37°C and analyzed for the amount of carotenoids, primary and secondary lipid oxidation products at week 0, 4, 8 and 12. The experimental set-up was exposed according to the *2x2* principle.

5.2.4. PREPARATION AND PROCESSING OF ENRICHED TOMATO PUREES

5.2.4.1. PREPARATION OF ENRICHED TOMATO PUREES

The different *Nannochloropsis* dosage forms (intact *Nannochloropsis* sp.*,* disrupted *Nannochloropsis* sp.*, Nannochloropsis* oil) or commercial fish oil were added to the thawed tomato puree in order to reach a concentration of 80 mg n-3 LC-PUFA/100 g puree. The incorporation concentrations for each of the dosage forms are shown in Table 5.1. The batch was mixed 2 times for 5 minutes, with a time interval of 5 minutes to avoid extensive heating of the sample, at 7500 rpm with a Silverson L5M-A lab mixer (East Longmeadow, MA, USA). The enriched tomato puree had a pH of 4.04 ± 0.12. The batch was divided into two parts: a part was labelled as '**mixed**' and a remaining part was further processed as described in section 5.2.4.2.

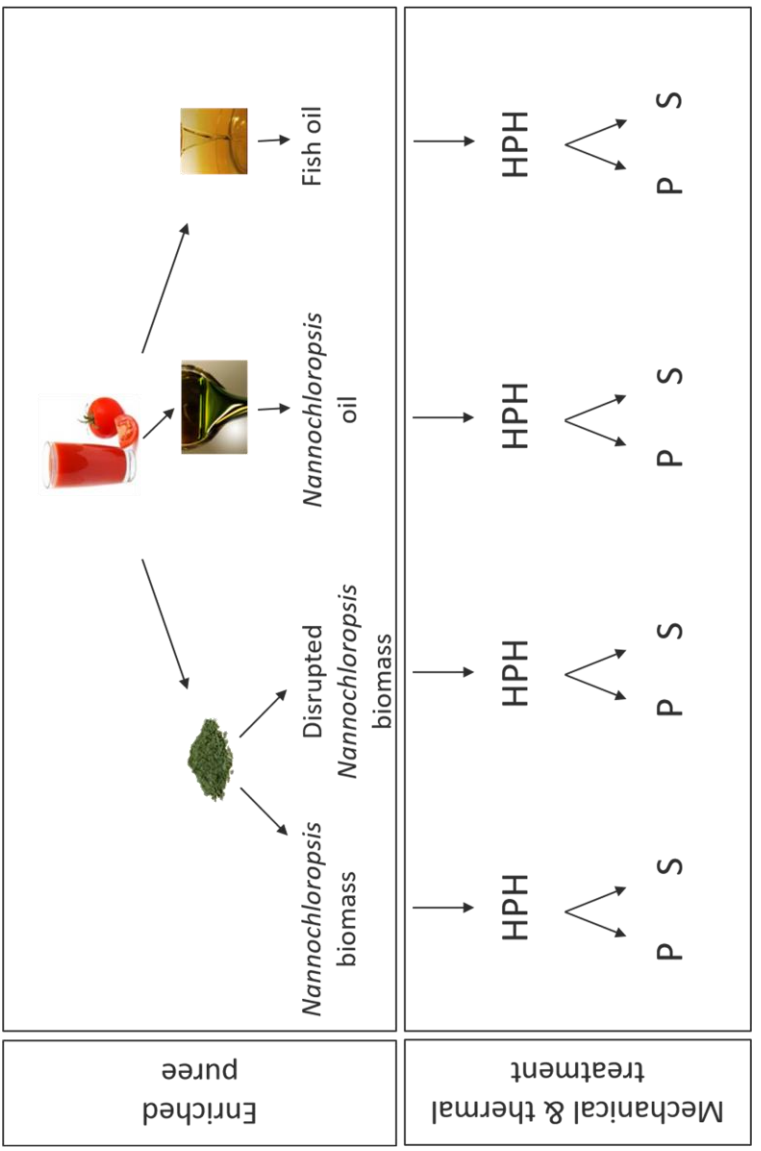


***Figure 5.1**. Experimental set-up of this chapter in which HPH stands for high pressure homogenization, P for pasteurization and S for sterilization. Each system was made in duplicate.*

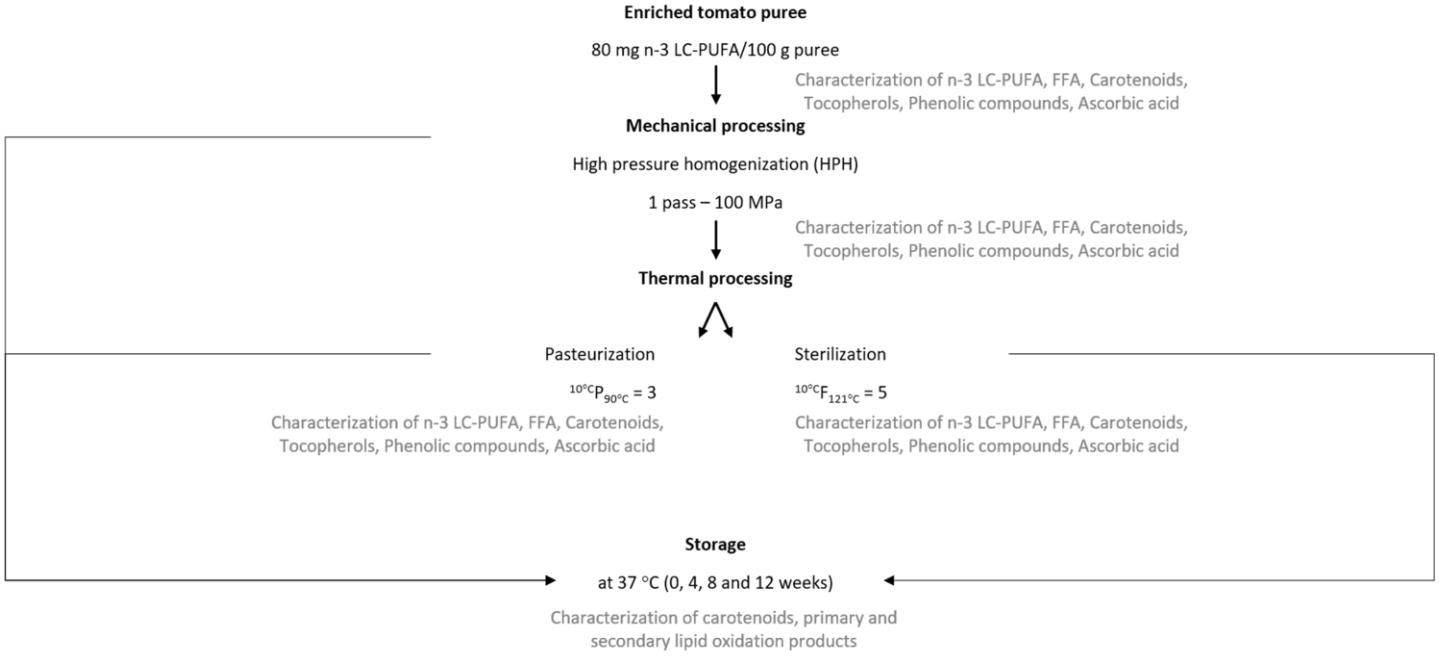


Figure 5.2. *Scheme of the different processing steps in the preparation process of enriched purees.*

5.2.4.2. MECHANICAL TREATMENT

The remaining part of the batch obtained in section 5.2.4.1 was high pressure homogenized (1 pass at 100 MPa) at 4°C. This high pressure homogenization step was applied to physically stabilize the homogenous puree and not necessarily to disrupt the microalgal cell (wall). The puree was collected in an ice bath. Again, the batch was divided into two parts: a part was labelled as '**high pressure homogenized**' and the remaining part underwent further thermal processing as described in section 5.2.4.3.

5.2.4.3. THERMAL TREATMENTS

Two different thermal treatments were performed. Therefore, the remaining enriched tomato puree obtained in section 5.2.4.2 was split between **pasteurization** ($^{10^{\circ}C}P_{90^{\circ}C}$ = 3 min) and **sterilization** ($^{10^{\circ}C}F_{121^{\circ}C}$ = 5 min). The thermal treatments were performed in glass jars (95 mm height, 45 mm diameter) with metal lids, each filled with 80 mL puree. Both thermal processes were performed in a pilot-scale water-cascading retort (Barriquand Steriflow, Paris, France). The temperature profiles were recorded, to control the treatment intensity, using thermocouples and registered by the Ellab Valsuite Plus software (Ellab, Hillerød, Denmark). Each puree was independently treated from his duplicate.

5.2.4.4. STORAGE

The treated enriched tomato purees (except for the fraction needed in the storage experiment) were freeze dried and stored at -80°C, until further analysis. High pressure homogenized, pasteurized and sterilized enriched tomato purees were involved in the storage experiment. The mixed purees were not stored, as their physical stability could not be guaranteed during 12 weeks of storage. The fraction needed for the accelerated storage test was chemically sterilized with 0.25% potassium sorbate. Amber screw-cap vials (20 mL) were filled with 7.5 mL treated puree, hermetically sealed and stored for 12 weeks at 37°C in an incubator (Advantage lab, Darmstadt, Germany). At this storage temperature an accelerated oxidation process was expected without

modifications in the oxidation pathways [156]. Four different time points (week 0, 4, 8 and 12) were evaluated in terms of carotenoids, primary and secondary oxidation. The vials for carotenoids and primary oxidation determination were freeze dried after the appropriate storage time. All vials were stored at -80°C until analysis, to prevent further oxidation. For each puree, a duplicate vial was stored.

5.2.5. ANALYSES

5.2.5.1. LIPID EXTRACTION

The lipids were extracted by a chloroform/methanol (1/1) extraction from the freeze dried puree as described in section 3.2.2.1. This analysis was performed in duplicate on each sample.

5.2.5.2. DETERMINATION OF N-3 LC-PUFA CONTENT

The n-3 LC-PUFA content was determined by chromatographic separation and FID detection after methylation of the extracted lipids according to the method described in section 4.2.3.2. The analysis was performed in duplicate.

5.2.5.3. DETERMINATION OF FREE FATTY ACID CONTENT

The total free fatty acid content was determined by chromatographic separation after the formation of diethylamide derivatives as described in section 4.2.3.4. The determination of total free fatty acid content was performed in duplicate.

5.2.5.4. DETERMINATION OF CAROTENOIDS

The carotenoid content was determined by HPLC-PAD with an optimized method based on the method described in section 4.2.3.5 [179,197]. In chapter 4, the β-carotene peak of thermal treated suspensions could not be quantified by overlap with chlorophyll degradation products. Moreover, lycopene is now present in the enriched purees. Therefore, in order to be able to extract, separate and quantify the different carotenoids, chlorophylls as well as their degradation products an optimized method was developed. Briefly, to 25 mg of freeze dried puree,

glass beads and 5 mL acetone/methanol (7/3) were added, after which the mixture was vortexed and centrifuged (10 min, 750 g, room temperature). The supernatant was kept and the steps described above were performed 3 more times. All extracts were pooled. The extract was filtered (PVDF syringe filters, 4 mm, 0.2 µm) and analyzed by HPLC coupled with photodiode array detection (PAD) at 436 nm (Alliance, Waters, Zellik, Belgium). The Nova Pak C18 column 60A (4 µm; 3.9 mm *x* 150 mm), was kept at 30°C and the samples at 10°C. The mobile phases methanol/ammonium acetate buffer (80/20, pH 7.2, 0.5 M) (A), acetonitrile/ultrapure water (90/10) (B) and ethyl acetate (C) were used in following gradient program:

Time (min)	Gradient
0-3	Linear from 100% A to 100% B
3-14	Linear from 100% B to 50% B and 50% C
14-16	Linear from 50% B and 50% C to 50% A and 50%
16-22.5	Linear from 50% A and 50% C to 20% A and 80% C
22.5-26	Isocratic at 20% A and 80% C
26-30	Linear from 20% A and 80% C to 100% C
30-32	Linear from 100% C to 80% A and 20% C
32-33	Linear from 80% A and 20% C to 100% A

External calibration curves were obtained for chlorophyll a, violaxanthin, antheraxanthin, zeaxanthin, lycopene and β-carotene (DHI, Horsholm, Denmark) to express the results as mg/100 g enriched puree. Peak areas were quantified with Empower 2 for Windows software (Waters, Zellik, Belgium). The determination of carotenoids was performed in duplicate.

5.2.5.5. DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

The determination of the phenolic content was performed with the Folin-Ciocalteu reagent according to the method of Goiris *et al.* [185]. Briefly, 2 mL ethanol/water (3/1) was added to 100 mg freeze dried puree. The samples were vortexed every 5 min, for 30 min, followed by centrifugation (10 min, 750 g, room temperature). The supernatant was transferred to a clean tube. The previous steps were repeated and both

extracts were pooled. 200 µL extract was mixed with 1.5 mL Folin-Ciocalteu reagent (previously diluted 10 times with water) and placed at room temperature. After 5 min, 1.5 mL sodium bicarbonate (60 g/L) solution was added. After an incubation time of 90 min, the mixture was spectrophotometrically measured at 750 nm. The blank of the reagent was measured by using 200 µL ethanol/water (3/1) instead of 200 µL extract. A calibration curve of gallic acid was set up by using 200 µL gallic acid (Carl Roth, Karlsruhe, Germany) solution (25, 50, 75, 100, 125 and 150 mg/L) instead of 200 µL extract. The determination of phenolic content was performed in duplicate.

5.2.5.6. DETERMINATION OF ASCORBIC ACID

The ascorbic acid content was determined by HPLC after a derivatization reaction according to Brown *et al.* [198]. Briefly, 4 mL meta-phosphoric acid solution and 20 µL EGTA-glutathione solution were added to 5 mg freeze dried puree. The mixture was vortexed 3 times and sonicated, followed by an incubation period of 1 h at 4°C in the dark. The mixture was centrifuged (10 min, 1000 g, 4°C) and 1.5 mL supernatant was kept to perform the derivatization. To the supernatant 0.4 mL sodium acetate buffer (pH 6.2) was added and the extract was incubated at 37°C. 10 min later, an ascorbic acid oxidase spatula was added for 5 min. After removing the spatula, 0.25 mL *o*-phenylene diamine (1 g/L) was added and further incubated at 37°C for 30 min. The derivatized extract was filtered before analyzing. Ascorbic acid standards (0.158 – 1.13 mg/L) (Carl Roth, Karlsruhe, Germany) were prepared and derivatized in the same way as the extracts. The standards and extracts were analyzed by HPLC and fluorescence detection (excitation at 335 nm, emission at 425 nm; Waters, Zellik, Belgium). The C18 column (Grace Altech Altima, 5 µm, 4.6 mm *x* 150 mm, Lokeren, Belgium), was kept at 30°C and the samples at 10°C. The mobile phase was 0.08 M Potassium phosphate/Methanol (8/2). Peak areas were quantified with Empower 2 for Windows software (Waters). The determination of ascorbic acid was performed in duplicate.

5.2.5.7. DETERMINATION OF TOCOPHEROLS

The amount of tocopherols was determined by HPLC after extraction according to Brown *et al.* [199]. Briefly, 5 mL ethanol, 0.5 mL 80% KOH and 50 mg ascorbic acid were added to 50 mg freeze dried puree. The mixture was vortexed and incubated for 30 min at 70°C. After cooling the mixture for 5 min at 0°C, 3 mL water and 5 mL hexane were added. After vortexing and centrifugation (10 min, 1000 g, 4°C), the hexane fraction was kept. To the remaining mixture, 5 mL fresh hexane was added. The mixture was vortexed and centrifuged and both hexane extracts were pooled. The extract was dried under nitrogen and the residue was dissolved in 1 mL methanol. The samples were analyzed by HPLC with fluorescence detection. Calibration was performed with α-tocopherol and peak areas were quantified with Empower 2 for Windows software (Waters). The determination of tocopherols was performed in duplicate.

5.2.5.8. DETERMINATION OF PRIMARY OXIDATION PRODUCTS

The primary oxidation products were analyzed spectrophotometrically by the ferrous oxidation xylenol orange method according to the method described in section 3.2.2.3. All data points were expressed as delta values, which is the relative increase or decrease compared to week zero. All analyses were done in duplicate.

5.2.5.9. DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Volatile secondary oxidation products were analyzed with HS-SPME GC-MS according to section 3.2.2.7. The semi quantitative concentrations of the identified volatile compounds were calculated as the area of the volatile indicator compound divided by the response factor of the external standard hexanal (spiked in a tomato based suspension). All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

5.2.6. CORRECTION FOR PHYSICAL LOSSES

All parameters were expressed per 100 g enriched puree. Physical losses during processing of the dosage forms by stickiness to glassware, mixer, tubes of high pressure homogenizer, etc. were corrected by using a correction factor, calculated on a fatty acid only present in the dosage forms and not in the tomato puree. Myristic acid (C14:0) was selected as the internal standard as it can be assumed that reduction of this saturated fatty acid can be only be attributed to physical losses. The following equation (5.1) was used:

$$Value_{CORRECTED} = Value_{MEASURED} x \frac{(C14{:}0)_{ADDED}}{(C14{:}0)_{MEASURED}} \quad (5.1)$$

This correction was performed on all values of compounds originating from the n-3 LC-PUFA source (lipids, antioxidants, primary and secondary oxidation compounds). Due to this correction, the purees could be compared relative to each other.

5.2.7. STATISTICAL ANALYSIS

Statistical comparison of the composition of the dosage forms was performed by a one way ANOVA for each of the parameters. Results of the amount of n-3 LC-PUFA, antioxidants and free fatty acids in the enriched tomato purees were statistically evaluated by a two way ANOVA to consider the impact of processing and dosage form. The oxidative stability (primary and secondary oxidation) was evaluated by two way ANOVA to consider the impact of time and dosage form for each of the processing types. The degradation of carotenoids (for each carotenoid individual) was evaluated by a one way ANOVA to consider the impact of time for each dosage form - processing intensity combination. Differences were determined by a *post hoc* Tukey test. All statistical tests were performed with $\alpha = 0.05$ (JMP Pro 12.1, SAS Institute Inc.).

5.3. RESULTS AND DISCUSSION

5.3.1. CHARACTERIZATION OF THE DOSAGE FORMS OF *NANNOCHLOROPSIS*

Three different dosage forms of *Nannochloropsis* were studied. Intact biomass referred to the biomass as such, without further pre-treatment before the incorporation in the tomato puree. Disrupted biomass was pre-treated by a high pressure homogenization (4 passes at 100 MPa) in order to (partially) disrupt the cell (wall) integrity. The difference in cell (wall) integrity between the intact and disrupted biomass was expressed as the extraction efficiency, which is the extraction yield obtained with hexane/isopropanol (3/2) compared to the extraction yield with chloroform/methanol (1/1) [66]. The disruption of the biomass increased the extraction efficiency from 45% for the intact biomass to 80% for disrupted biomass. This increase in extraction efficiency indicated that the cell (wall) was at least partially disrupted and thus that the cell (wall) integrity of the disrupted *Nannochloropsis* was lower compared to the intact *Nannochloropsis*. Figure 5.3 shows also the impact of high pressure homogenization on the extent of cell (wall) integrity by differential interference contrast microscopy and scanning electron microscopy. The images of the intact biomass (Figure 5.3a) show cells with a clear cell surface, while the cells in the images of the disrupted biomass (Figure 5.3b) were damaged and aggregates of released compounds could be observed, although intact cells could still be observed in the pre-treated biomass. These images confirm that the high pressure homogenization indeed resulted in a partial disruption of the cell (wall) integrity. To get more insight in the impact of high pressure homogenization on the intactness of microalgal cell organelles, transmission electron microscopy as proposed by Halim *et al.* [200] could be performed on the disrupted microalgal biomass. The third dosage form was *Nannochloropsis* oil, obtained by a large scale hexane/isopropanol (3/2) extraction.

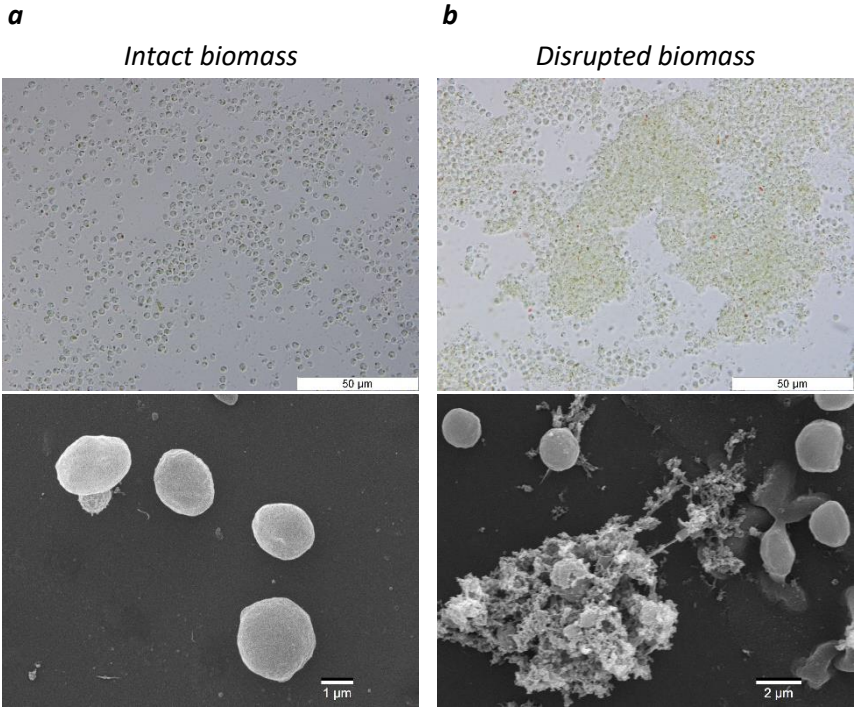


Figure 5.3. *Representative differential interference contrast microscopy (upper) and scanning electron microscopy (lower) images of intact Nannochloropsis biomass (a) and disrupted Nannochloropsis biomass (b). The images are owned by Tom Bernaerts (LFT, KULeuven).*

The three different dosage forms of *Nannochloropsis* contained different concentrations of n-3 LC-PUFA (Table 5.1). *Nannochloropsis* oil contained significantly more n-3 LC-PUFA/g compared to intact and disrupted *Nannochloropsis* biomass. This is due to the increase in concentration of n-3 LC-PUFA in the former dosage form that occurs during lipid extraction. Moreover, differences in free fatty acid and antioxidant content could be observed. Pre-treatment of *Nannochloropsis* increased the amount of free fatty acids significantly. The highest concentration of free fatty acids was observed in *Nannochloropsis* oil, followed by disrupted *Nannochloropsis* and intact *Nannochloropsis,* respectively. Smaller, but significant differences were observed in the antioxidant composition. No difference in carotenoid content between intact and disrupted biomass

was observed, while *Nannochloropsis* oil had significantly higher antheraxanthin content. The highest phenolic and tocopherol concentrations were also observed in the latter. Ascorbic acid was significantly more present in the intact biomass, compared to the disrupted biomass and compared to the *Nannochloropsis* oil.

The difference in n-3 LC-PUFA content in the different dosage forms resulted in a different incorporation concentration in order to reach a fixed concentration of 80 mg n-3 LC-PUFA/100 g tomato puree. This implies that when comparing the enriched tomato purees, differences could not only be explained by differences in composition but also by differences in incorporation concentration.

The main differences observed between the *Nannochloropsis* dosage forms and the commercial fish oil were the absence of carotenoids and the higher content of n-3 LC-PUFA in the latter. The high tocopherol concentration in commercial fish oil could be explained by the addition of tocopherols during the production process of commercial fish oil.

5.3.2. N-3 LC-PUFA CONTENT OF ENRICHED TOMATO PUREE

The different n-3 LC-PUFA sources were incorporated in the tomato purees in concentrations varying between 0.49 and 2.12% (Table 5.1) in order to reach a fixed concentration of 80 mg n-3 LC-PUFA/100 g enriched puree. It is worth mentioning that physical losses of n-3 LC-PUFA up to 50% were observed in purees supplemented with *Nannochloropsis* oil. These losses occurred during the mixing and high pressure homogenization step by stickiness to glassware, mixer, tubes of high pressure homogenizer, etc. The physical losses of the purees supplemented with *Nannochloropsis* oil were 5 times higher compared to the purees supplemented with other dosage forms. These physical losses resulted in the fact that the label 'rich in omega-3' could not be claimed anymore in the processed puree. Higher incorporation concentrations are thus needed to obtain the desired final concentration of 80 mg n-3 LC-PUFA/100 g enriched puree, which will lead to a higher cost. Therefore, from a practical and sustainability point of view, a lower

usability of *Nannochloropsis* oil as dosage form was concluded. Throughout this chapter all values were corrected for these physical losses (as described in section 5.2.6) in order to be able to actually compare the dosage forms on a standardized amount of n-3 LC-PUFA.

Figure 5.4 shows the amount of n-3 LC-PUFA corrected for physical losses in the tomato purees supplemented with the different n-3 LC-PUFA sources after each processing step. As expected, no significant differences between the n-3 LC-PUFA sources were observed in the mixed purees as the initial amount of n-3 LC-PUFA was standardized and corrected to a concentration of 80 mg n-3 LC-PUFA/100 g enriched puree (section 5.2.6). Within each of the n-3 LC-PUFA sources, no impact of mechanical (high pressure homogenization) or thermal processing (pasteurization or sterilization) on the remaining amount of n-3 LC-PUFA was detected.

Similar results were obtained in the research of Gouveia *et al.* [76] and Fradique *et al.* [77], who investigated the impact of processing on n-3 LC-PUFA concentration in food products supplemented with photoautotrophic microalgae. No significant impact of cooking (3-5 min in boiling water) the pasta or heating the dessert to gelling temperatures (5 min at 90°C) on the n-3 LC-PUFA concentration was reported. Similarly, Alejandre *et al.* [103] observed no impact of processing in patties supplemented with 0.1% heterotrophic microalgal oil. However, contradictory results were obtained in the work of Lee *et al.* [94]. Sausages supplemented with heterotrophic microalgal oil, showed losses of n-3 LC-PUFA up to 20% due to cooking. Also in chapter 4, a reduction of n-3 LC-PUFA by processing when microalgal biomass was added to an aqueous model system was observed. However, none of the previously mentioned studies were executed on enriched fruit and vegetable-based products. The observed differences with literature can likely be explained by a protective role of the tomato puree.

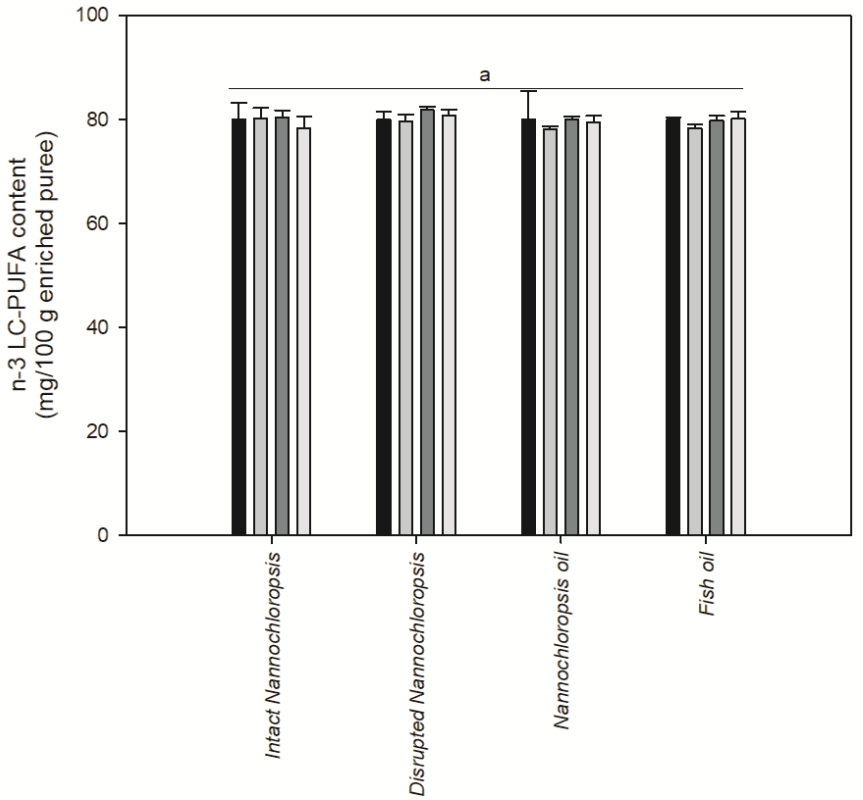


Figure 5.4. *Amount of n-3 LC-PUFA (expressed as mg/100 g enriched puree) after correction for physical losses in tomato puree supplemented with intact Nannochloropsis biomass, disrupted Nannochloropsis biomass, Nannochloropsis oil or fish oil after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●). No significant differences were observed, α=0.05.*

5.3.3. OXIDATIVE STABILITY OF ENRICHED TOMATO PUREE

5.3.3.1. LIPID OXIDATION

The formation of primary oxidation and secondary oxidation products was investigated during storage at 37°C for 12 weeks (Figure 5.5). Purees supplemented with fish oil showed, independent of the processing steps performed, the highest degree of oxidation as an increase in the amount of hydroperoxides was followed by a decrease upon further storage. The significant increase observed between week 0 and 4 was followed by a

significant decrease in the pasteurized and sterilized tomato puree. Although the decrease was not significant in the high pressure homogenized puree, the same trend could be observed visually. This means that the maximum amount of hydroperoxides was already obtained during the storage period after which the primary oxidation products were transformed faster into secondary oxidation products than they were formed. This was confirmed by the fact that the volatile compounds, as a marker for the secondary oxidation, increased significantly over the storage time.

Purees supplemented with *Nannochloropsis* dosage forms performed significantly better than purees supplemented with fish oil. Only small, although significant, differences in oxidative stability were observed between the purees supplemented with different *Nannochloropsis* dosage forms. Purees supplemented with *Nannochloropsis* oil showed the lowest oxidative stability of the purees supplemented with n-3 LC-PUFA derived from the microalga *Nannochloropsis*. Already in week 4, a significant increase in the amount of hydroperoxides was detected for all types of processing, indicating lipid oxidation. This observation was confirmed by the significant increase in the volatile secondary oxidation compounds during storage. Again no differences between the purees supplemented with *Nannochloropsis* oil after different processing steps could be established. Purees supplemented with intact and disrupted *Nannochloropsis* biomass showed the lowest amount of oxidation products formed during storage. Both dosage forms resulted in a zero to limited increase in primary (hydroperoxides, earliest at week 4) and in secondary (selected volatiles, earliest at week 8) oxidation compounds. Small differences between the dosage forms were observed, indicating the potential of the use of (partially) disrupted biomass to improve the bioaccessibility without reduction of the oxidative stability.

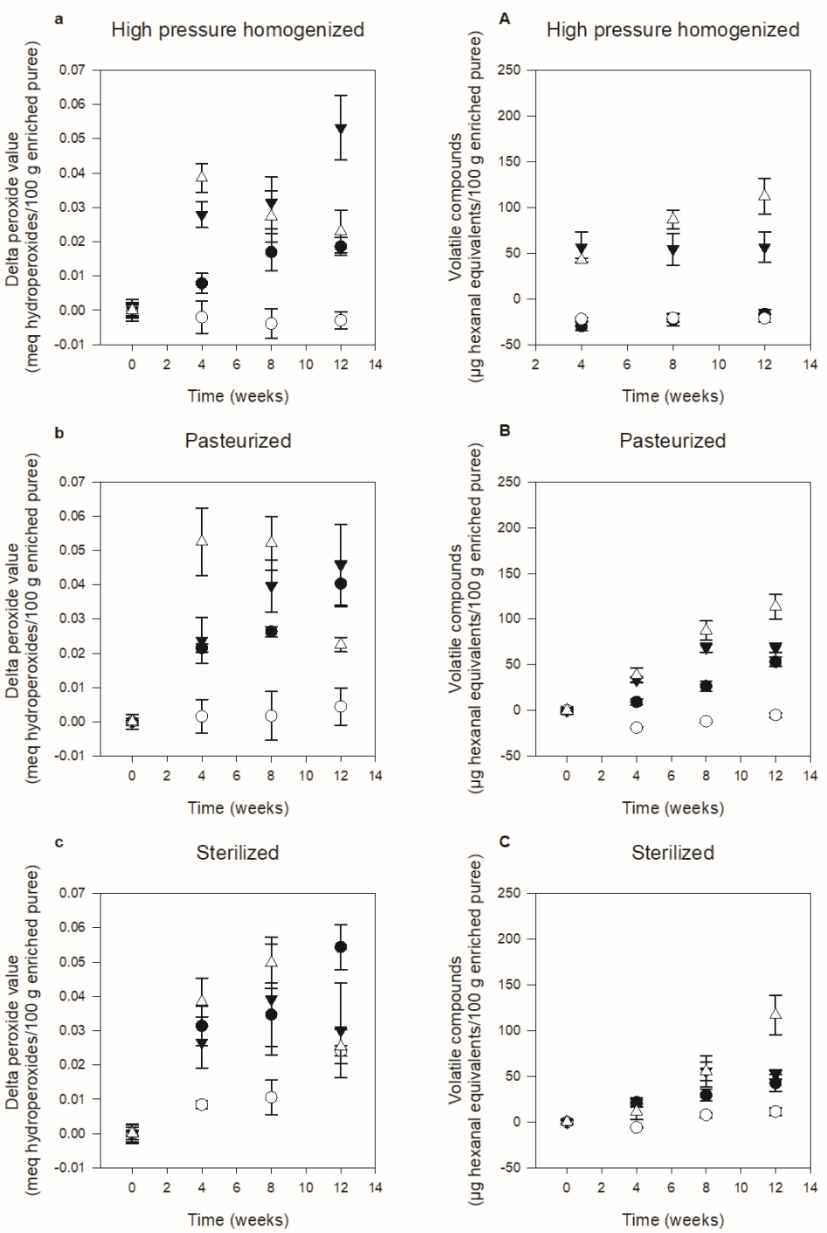


Figure 5.5. *Evolution of primary (small letter) and secondary (capital letter) oxidation products during 12 weeks of storage at 37°C for tomato purees supplemented with intact Nannochloropsis biomass (●), disrupted Nannochloropsis biomass (○), Nannochloropsis oil (▼) and fish oil (Δ) after high pressure homogenization (a & A), pasteurization (b & B) and sterilization (c & C), , mean (± SD). The results are expressed as delta values, which is the relative increase or decrease compared to week zero.*

A significant reduction in the volatile compounds between week 0 and week 4 was observed in high pressure homogenized purees supplemented with *Nannochloropsis* biomass. This might be explained by the presence of algal volatile organic compounds (AVOC) formed *in vivo* and still present at week 0 [201]. Based on literature, it can be suggested that these compounds were decomposed during storage (between week 0 and 4) or during thermal processing [202], which explains the absence of this decrease in the pasteurized and sterilized samples. AVOC were probably also completely removed during the preparation of the *Nannochloropsis* oil dosage form explaining why no decrease between week 0 and 4 could be observed in any of the samples supplemented with *Nannochloropsis* oil. In an additional storage experiment, the same *Nannochloropsis* biomass was mixed in an aqueous suspension (without any further mechanical or thermal treatment) in analogy with the processing step described in section 5.2.4.1. In this enriched aqueous suspension the oxidation markers (peroxide value and volatile compounds) increased drastically during storage, which implies that the biomass was not yet oxidized at the beginning of the storage experiment (data not shown). Thus extensive oxidation could not be the causative factor for the volatiles observed at week 0. This makes the hypothesis of AVOC reliable. In contrast to the high impact of the dosage form used, the impact of processing on the oxidative stability was very limited and no conclusive results could be drawn. Only the purees supplemented with (intact or disrupted) biomass showed a slight increased degree of oxidation due to pasteurization and sterilization.

From the above, it is clear that purees supplemented with *Nannochloropsis* performed better in terms of oxidative stability than purees supplemented with fish oil. Many factors can influence the rate of lipid oxidation: the amount of carotenoids, phenolic compounds, ascorbic acid, tocopherols and free fatty acids. These possible causative factors are further described in section 5.3.3.2 and their relation with the oxidative stability is discussed in section 5.3.3.3.

5.3.3.2. INFLUENCING FACTORS

i. *CAROTENOIDS*

Photoautotrophic microalgae as well as tomatoes deliver different carotenoids to the enriched purees [203,204]. Figure 5.6(a, b, c) shows the amount of carotenoids expressed per 100 g enriched puree. Lycopene present in the purees originated from the tomato and the amounts (5 mg/100 g enriched puree) were therefore independent of the used n-3 LC-PUFA source.

β-carotene was observed in all enriched purees due to the fact that tomato puree as well as the *Nannochloropsis* dosage forms contained β-carotene. In all purees supplemented with a dosage form of *Nannochloropsis*, additional carotenoids (zeaxanthin, antheraxanthin and minor amounts of violaxanthin) were observed. These three carotenoids contain the same backbone structure. Zeaxanthin contains a double bound in each of the cyclic end structures, while antheraxanthin and violaxanthin contain respectively one and no double bound in the end structures. This implies that zeaxanthin has the highest antioxidant potential followed by antheraxanthin and violaxanthin [130]. In addition, zeaxanthin – antheraxanthin and antheraxanthin – violaxanthin can be reversely converted into each other via an enzymatic pathway [205].

The amount of β-carotene, zeaxanthin and antheraxanthin in the purees supplemented with the different n-3 LC-PUFA sources after mixing were in line with the calculated expectations based on the amount of the carotenoids measured in the n-3 LC-PUFA sources (Table 5.1 and 5.2). This observation indicated that differences in the carotenoid concentration in the purees could be ascribed to a combined effect of differences in the incorporation concentration as well as in the initial amounts present in the n-3 LC-PUFA source.

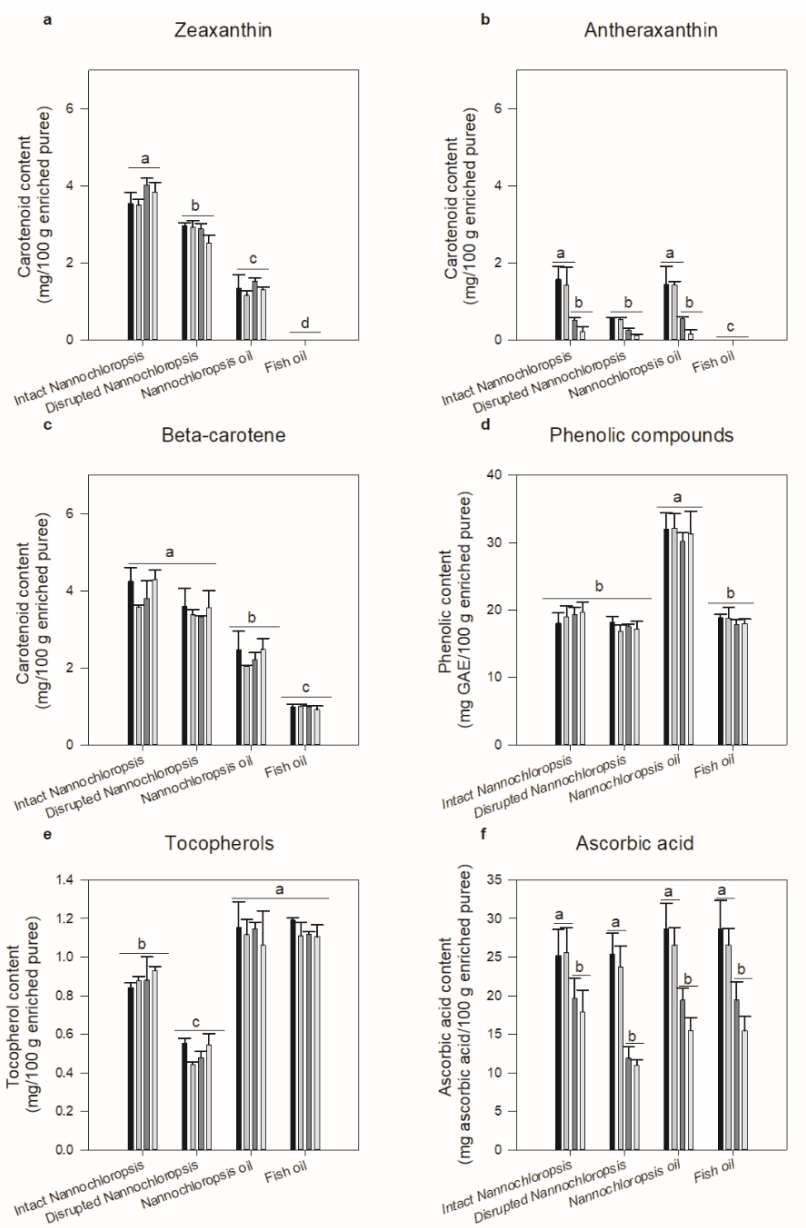


Figure 5.6. *Carotenoid composition, zeaxanthin (a), antheraxanthin (b) and β-carotene (c), and phenolic compounds (e), tocopherols (e) and ascorbic acid (f) expressed as mg/100 g enriched puree, for tomato purees supplemented with intact Nannochloropsis biomass, disrupted Nannochloropsis biomass, Nannochloropsis oil and fish oil after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

Table 5.2. *Theoretical calculated values of n-3 LC-PUFA, free fatty acids and antioxidants (mg/100 g enriched puree) of tomato puree enriched with intact Nannochloropsis biomass, disrupted Nannochloropsis biomass, Nannochloropsis oil and fish oil, mean (± SD).*

	Intact *Nannochloropsis*	**Disrupted *Nannochloropsis***	***Nannochloropsis* oil**	**Fish oil**
n-3 LC-PUFA (mg/100 g enriched puree)	80 (± 1.1)	80 (± 2)	79.9 (± 1.2)	79.5 (± 0.7)
Free fatty acid (mg/100 g enriched puree)	24 (± 3)	58.9 (± 1.9)	44 (± 3)	4.5 (± 0.3)
Carotenoids (mg/100 g enriched puree)				
Antheraxanthin	1.6 (± 0.3)	0.59 (± 0.02)	1.4 (± 0.4)	-
Zeaxanthin	3.5 (± 0.3)	2.97 (± 0.06)	1.3 (± 0.3)	-
β-carotene	4.2 (± 0.5)	3.5 (± 0.6)	2.4 (± 0.8)	0.95 (± 0.01)
Lycopene	5.13 (± 0.02)	5.13 (± 0.02)	5.13 (± 0.02)	5.13 (± 0.02)
Phenolic compounds (mg/100 g enriched puree)	24.9 (± 0.8)	25.3 (± 0.9)	38 (± 3)	19 (± 2)
Ascorbic Acid (mg/100 g enriched puree)	28 (± 2)	26 (± 2)	25 (± 4)	25 (± 4)
Tocopherols (mg/100 g enriched puree)	0.97 (± 0.04)	0.49 (± 0.12)	1.39 (± 0.03)	1.23 (± 0.04)

Fish oil did not contain β-carotene itself and therefore all β-carotene (1 mg β-carotene/100 g enriched puree) originated from the tomato puree in samples supplemented with fish oil. Purees supplemented with *Nannochloropsis* biomass contained 4 mg β-carotene/100 g enriched puree and purees supplemented with *Nannochloropsis* oil 2.4 mg β-carotene/100 g enriched puree. Zeaxanthin was the main carotenoid originating from the *Nannochloropsis* dosage forms with 3.7 mg zeaxanthin/100 g enriched puree in the case of intact biomass. Reductions in the amount of zeaxanthin were observed by the use of the different dosage forms. Purees supplemented with disrupted biomass only contained 3 mg zeaxanthin/100 g enriched puree. Table 5.1 indeed suggested a reduction in the amount of zeaxanthin by the supplemental disruption step considering the same incorporation concentration of both biomass dosage forms. Purees supplemented with *Nannochloropsis* oil only contained 1.3 mg zeaxanthin/100 g enriched puree, which can be explained by the three times lower incorporation concentration to obtain the same amount of n-3 LC-PUFA/100 g enriched puree. Antheraxanthin was observed in all purees supplemented with intact *Nannochloropsis, Nannochloropsis* oil or disrupted *Nannochloropsis*, with values of 1.5, 1.3 and 0.6 mg antheraxanthin/100 g enriched puree respectively. Again the different incorporation concentrations used and the amount present in the initial dosage form, explained the amounts in the enriched purees. Violaxanthin (results not shown) was only present in purees supplemented with biomass, with a maximum amount of 0.17 mg violaxanthin/100 g puree in the purees supplemented with intact biomass.

In none of the purees the amounts of lycopene, β-carotene or zeaxanthin were influenced by the different processing steps, being in accordance to literature [204]. Thermal processing drastically reduced the amount of antheraxanthin in purees supplemented with intact biomass and oil. This difference was not significant in the purees supplemented with disrupted biomass, although a decreasing trend in antheraxanthin by thermal processing could be visually observed. Violaxanthin was not detectable after thermal treatment.

Overall, purees supplemented with intact *Nannochloropsis* biomass contained the highest amount of carotenoids, followed by purees supplemented with disrupted *Nannochloropsis* biomass and *Nannochloropsis* oil and lastly followed by purees supplemented with fish oil. Lycopene, β-carotene and zeaxanthin, the latter absent in purees supplemented with fish oil, were the main carotenoids present in the purees. Lycopene, β-carotene and zeaxanthin are lipid soluble free radical scavengers, of which in literature zeaxanthin is described as the better antioxidant [124].

In addition, chlorophyll a, another pigment (although not a carotenoid) originating from *Nannochloropsis*, was observed in low amounts (2 mg chlorophyll a/100 g puree) in the mixed purees supplemented with intact biomass. In all other purees supplemented with *Nannochloropsis*, only degradation products of chlorophyll a, formed during processing and preparation of the dosage forms, could be detected (results not shown). Chlorophyll a and chlorophyll a degradation products are well-known pro-oxidants under light exposed storage, although in dark conditions, as is the case in this chapter, antioxidant effects are attributed to these compounds [140]. However, the presence of these compounds is of inferior importance compared to carotenoids in the maintenance of the oxidative stability.

ii. *TOTAL PHENOLIC COMPOUNDS*

Phenolic compounds were present in the purees as well (Figure 5.6d). Significantly more phenolic compounds could be observed in the purees supplemented with *Nannochloropsis* oil (32 mg phenolic compounds/100 g puree), despite the lower incorporation concentrations, compared to the others (18 mg phenolic compounds/100 g puree). An enrichment of the phenolic compounds during extraction of the oil and thus a higher concentration in the dosage form explained this observation (Table 5.1 and 5.2). Furthermore, no differences between the enriched purees could be detected, as the largest part of the phenolic compounds originated from the tomato puree. In addition, no impact of processing was

observed. Dewanto *et al.* [206] already showed a high thermal stability of phenolic compounds originating from tomatoes.

iii. *TOCOPHEROLS*

Tocopherols (vitamin E) (Figure 5.6e) originated from the *Nannochloropsis* (naturally rich in tocopherols) or commercial fish oil (supplemented with tocopherols) as well as from the tomato puree. Purees supplemented with *Nannochloropsis* and fish oil showed the highest tocopherol concentrations (1.1 mg tocopherols/100 g puree). Despite the lower incorporation concentration of *Nannochloropsis* oil, the high oil solubility of tocopherols led to an increase in the concentration during extraction of the oil [124], resulting in the highest tocopherol concentration in purees supplemented with *Nannochloropsis* oil. A lower amount of tocopherols, 0.9 mg tocopherols/100 g puree, was detected in the purees supplemented with intact biomass and the lowest amount was observed in purees supplemented with disrupted biomass (0.5 mg tocopherols/100 g puree). The lower tocopherol concentration in purees supplemented with disrupted biomass can be explained by the significant loss during the pre-treatment to disrupt the biomass (Table 5.1). In none of the samples a significant impact of processing could be observed.

iv. *ASCORBIC ACID*

Ascorbic acid (vitamin C) was also present in the purees and mainly originated from the tomato puree. This explains why no differences in ascorbic acid concentration between the purees supplemented with different n-3 LC-PUFA sources were observed (Figure 5.6f). Initially 25 mg ascorbic acid/100 g puree was present but this amount was highly reduced by thermal processing. In this chapter, a reduction of 30% was detected, although no significant impact of the intensity of the heat treatment was observed (pasteurization versus sterilization). These results are in accordance with the results of Sanchez-Moreno *et al.* [207].

v. *FREE FATTY ACIDS*

Another factor that may affect the oxidative stability, is the presence of free fatty acids [208]. Moreover, the presence of free fatty acids should be avoided since they may result in a rancid flavor and pro-oxidative effects. Figure 5.7 presents the total amount of free fatty acids in the tomato purees supplemented with different *Nannochloropsis* dosage forms or fish oil. Significant differences between the purees supplemented with different *Nannochloropsis* dosage forms and fish oil were detected, which could be ascribed to the differences in the free fatty acids content of the n-3 LC-PUFA source and the incorporation concentration (Table 5.1 and 5.2). Purees supplemented with commercial fish oil showed a significantly lower amount of free fatty acids. Commercial fish oil is refined, in which most of the free fatty acids have been removed. This led to a total amount of 10 mg free fatty acids/100 g enriched puree. In purees supplemented with *Nannochloropsis* dosage forms, significantly more free fatty acids were observed. The purees supplemented with intact biomass showed more free fatty acids (35 mg/100 g enriched puree) compared to purees supplemented with fish oil. Purees supplemented with disrupted biomass and *Nannochloropsis* oil showed even more free fatty acids compared to purees supplemented with intact biomass, respectively 55 and 40 mg/100 g enriched puree. The same incorporation concentration of disrupted *Nannochloropsis* biomass and almost 3 times less *Nannochloropsis* oil was used per 100 g puree compared to intact *Nannochloropsis* biomass. It is therefore proposed that increased lipase activity during the additional disruption of the biomass and the extraction of the oil caused the formation of free fatty acids (Table 5.1). Processing of the enriched purees did not influence the amount of free fatty acids.

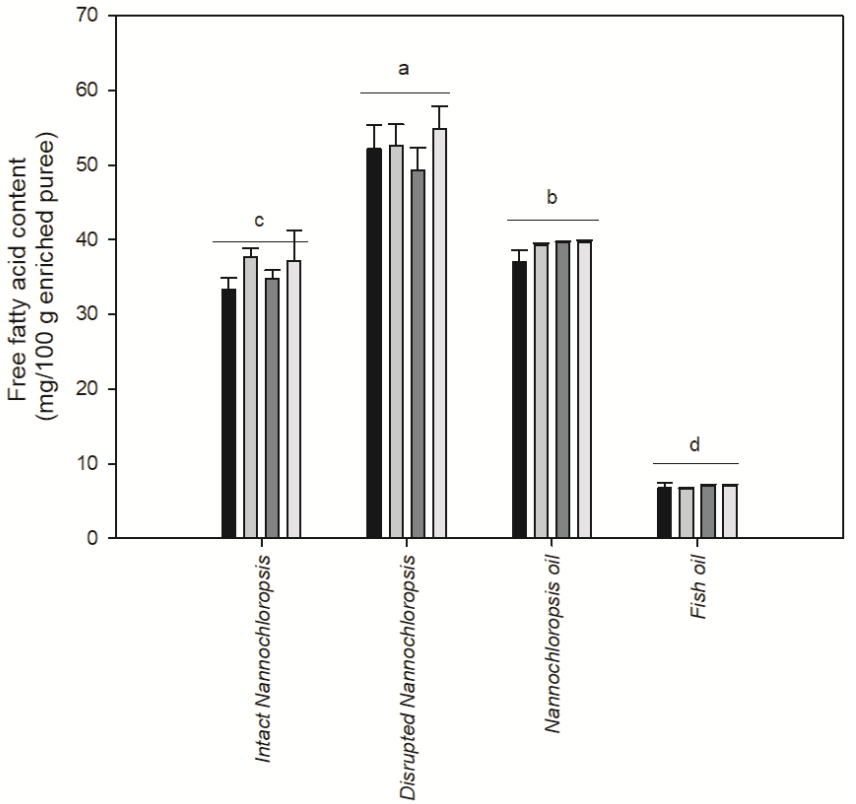


Figure 5.7. *Free fatty acid content, expressed as mg/100 g enriched puree, for tomato purees supplemented with intact Nannochloropsis biomass, disrupted Nannochloropsis biomass, Nannochloropsis oil and fish oil after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

5.3.3.3. RELATION BETWEEN OXIDATIVE STABILITY AND INFLUENCING FACTORS

Large differences in oxidative stability between the purees supplemented with different *Nannochloropsis* dosage forms and with fish oil were observed (5.3.3.1). In parallel, differences in factors known for their impact on the oxidative stability were seen (5.3.3.2).

The carotenoids (β-carotene, zeaxanthin and antheraxanthin) may have fulfilled an important role in the suppression of lipid oxidation, as they were present in larger concentrations in purees with a higher oxidative stability. On the other hand, the role of tocopherols, ascorbic acid and

phenolic compounds in the maintenance of the oxidative stability appears to be minor considering the higher concentrations in the purees with a lower oxidative stability. Interactions between the different antioxidants can also not be excluded [124]. Pro-oxidative free fatty acids were not the determining factor for the differences in the oxidative stability, since the lowest amount of free fatty acids was observed in the purees with the lowest oxidative stability. Purees supplemented with intact or (partially) disrupted biomass showed a higher oxidative stability compared to purees supplemented with oil. The cell (wall) integrity was therefore not determinative for maintenance of the oxidative stability as no differences worth mentioning between intact and (partially) disrupted biomass were observed. Intact and (partially) disrupted biomass both contained, besides higher concentrations of endogenous carotenoids, microalgal cell wall material. In literature, protein hydrolysates and peptides, polysaccharides, plant based sterols,… are described as novel antioxidants [209]. Possibly these compounds are present in the microalgal cell wall as well. However, to substantiate this hypothesis further research should be undertaken. In summary, it was thus hypothesized that the higher oxidative stability of tomato puree supplemented with *Nannochloropsis* biomass could primarily be attributed to the presence of higher concentrations of carotenoids.

In order to support this hypothesis, the evolution of the carotenoids during the 12 weeks storage experiment was investigated. Tables 5.3a−c show the evolution of the main carotenoids (> 1 mg/100 g enriched puree at week zero in at least one of the enriched purees) originating from tomatoes (lycopene and a part of the β-carotene) and originating from the microalgae (zeaxanthin, antheraxanthin and a part of the β-carotene).

In general, all carotenoids showed a decrease over time in all enriched purees, indicating that during storage the carotenoids degraded. Degradation of carotenoids can be caused by their scavenging of peroxyl radicals, by scavenging oxygen (singlet or triplet), by light and by high temperature [210]. Light and high temperature can be excluded as causative factors as the storage was performed in darkness at 37°C. This implies that

the degradation of carotenoids was caused by the reaction with radicals or oxygen. In both cases the carotenoids help to prevent further lipid oxidation and thus exert an antioxidative role. The reaction of carotenoids with radicals breaks the autoxidation chain reaction, while reaction with oxygen helps to prevent the formation of new oxidized lipids [211].

In purees enriched with *Nannochloropsis*, irrespective of the dosage form or processing intensity, a significant degradation of zeaxanthin was observed from week 4. Reductions up to 89% were observed. Additionally, antheraxanthin was in all purees, except for the high pressure homogenized puree enriched with *Nannochloropsis* oil, completely degraded at week 4. β-carotene in purees enriched with *Nannochloropsis* showed significant degradations from week 4 and a total reduction up to 75% after 12 weeks of storage. It could however not be observed if these reductions were more pronounced in the β-carotene derived from *Nannochloropsis* or from the tomato puree. Purees enriched with fish oil contained lower amounts of β-carotene, as it was only derived from the tomato puree. Significant decreases from week 4 and degradations up to 85% were seen with almost no β-carotene left. Large differences in lycopene (from the tomato puree) degradation enriched with *Nannochloropsis* dosage forms or fish oil were observed. Lycopene showed a limited degradation up to 45% during the storage period in purees enriched with *Nannochloropsis* biomass, while the degradation was more pronounced in tomato purees enriched with *Nannochloropsis* oil and fish oil (up to 75%). Additionally, the significant degradation occurred already in an earlier stage of the storage experiment in the case of the latter. The lycopene reduction suggests an antioxidant activity although this was insufficient to maintain the oxidative stability given the fact that the fish oil started to oxidize from week 4.

These results suggest that if carotenoids derived from photoautotrophic microalgae are present in sufficient amount, they degrade preferentially to act as antioxidant compared to carotenoids derived from tomato puree. If only limited amounts of carotenoids derived from microalgae are present, the carotenoids from the tomato puree degrade more

intensively. Purees enriched with fish oil did not contain carotenoids derived from microalgae and this resulted in higher lycopene reductions.

Furthermore, it was observed that if the purees enriched with *Nannochloropsis* biomass were more intensely thermally processed, the endogenous carotenoid concentrations decreased more during storage. This coincides with the observation that purees supplemented with (intact or disrupted) biomass showed a slightly increased degree of oxidation due to pasteurization and sterilization. Although no differences in carotenoid concentration could be observed at week zero, the formation of higher amounts of reactive oxygen species during thermal processing may have led to the higher carotenoids degradation during storage and thus the slightly more pronounced oxidation [212]. This could not be observed in purees enriched with *Nannochloropsis* oil or fish oil, possibly because the amount of carotenoids derived from the microalgae was already limited in the high pressure homogenized purees at week zero.

All these observations support the hypothesis that mainly the carotenoids, and more specifically, zeaxanthin, antheraxanthin and β-carotene from the microalgae helped to improve the oxidative stability. Most likely, their stronger antioxidant capacity compared to lycopene and/or their closer location to the n-3 LC-PUFA (when using photoautotrophic microalgal biomass or oil, carotenoids and n-3 LC-PUFA are located in the same cell (or even cell organelle), or in the same oil droplet) leads to the higher oxidative stability of vegetable purees enriched with photoautotrophic microalgae.

Table 5.3. *Evolution of the main carotenoids (> 1 mg/100 g enriched puree) of purees supplemented with intact Nannochloropsis biomass, disrupted Nannochloropsis biomass, Nannochloropsis oil and fish oil after high pressure homogenization (a), pasteurization (b) and sterilization (c) during 12 weeks of storage, mean (± SD). Statistical differences in function of time (per enriched puree) are indicated with a different letter, α=0.05.*

a	Dosage form	High pressure homogenized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	Intact *Nannochloropsis*	4.9 (± 0.3)^{a}	3.7 (± 0.3)^{a}	4.2 (± 0.4)^{a}	3.8 (± 0.2)^{a}
	Disrupted *Nannochloropsis*	4.8 (± 0.2)^{a}	4.53 (± 0.18)^{a}	4.4 (±0.7)^{a}	3.85 (± 0.10)^{a}
	Nannochloropsis oil	5.06 (± 0.05)^{a}	5.0 (± 1.7)^{ab}	4.0 (±1.3)^{b}	1.6 (± 0.5)^{c}
	Fish oil	5.34 (± 0.06)^{a}	3.1 (± 0.3)^{b}	2.2 (±0.2)^{c}	1.6 (± 0.2)^{c}
β-carotene	Intact *Nannochloropsis*	3.57 (± 0.05)^{a}	2.4 (± 0.2)^{b}	2.2 (± 0.5)^{b}	2.04 (± 0.15)^{b}
	Disrupted *Nannochloropsis*	3.38 (± 0.13)^{a}	3.3 (± 0.2)^{a}	2.7 (± 0.2)^{ab}	2.09 (± 0.16)^{b}
	Nannochloropsis oil	2.04 (± 0.03)^{a}	2.6 (± 1.0)^{a}	1.6 (± 0.5)^{a}	0.63 (± 0.17)^{a}
	Fish oil	1.02 (± 0.05)^{a}	0.33 (± 0.04)^{b}	0.19 (± 0.03)^{bc}	0.15 (± 0.02)^{c}
Zeaxanthin	Intact *Nannochloropsis*	3.5 (± 0.2)^{a}	1.64 (± 0.15)^{b}	1.6 (± 0.4)^{b}	1.31 (± 0.15)^{b}
	Disrupted *Nannochloropsis*	2.93 (± 0.06)^{a}	1.57 (± 0.14)^{b}	1.33 (± 0.08)^{bc}	1.03 (±0.03)^{c}
	Nannochloropsis oil	1.16 (± 0.15)^{a}	0.8 (± 0.6)^{ab}	0.7 (± 0.4)^{ab}	0.29 (± 0.07)^{b}
	Fish oil	-	-	-	-
Antheraxanthin	Intact *Nannochloropsis*	1.42 (± 0.19)	-	-	-
	Disrupted *Nannochloropsis*	0.53 (± 0.13)	-	-	-
	Nannochloropsis oil	1.44 (± 0.09)^{a}	0.3 (± 0.2)^{b}	0.22 (± 0.12)^{b}	0.14 (± 0.05)^{b}
	Fish oil	-	-	-	-

b

	Dosage form	Pasteurized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	Intact *Nannochloropsis*	4.8 (± 0.3)^{a}	3.71 (± 0.12)^{b}	2.99 (± 0.13)^{bc}	2.7 (± 0.4)^{c}
	Disrupted *Nannochloropsis*	5.0 (± 0.3)^{a}	3.5 (± 0.4)^{b}	3.5 (± 0.2)^{b}	2.2 (± 0.3)^{c}
	Nannochloropsis oil	5.56 (± 0.04)^{a}	4.3 (± 0.2)^{b}	2.1 (± 0.3)^{c}	1.4 (± 0.2)^{c}
	Fish oil	5.5 (± 0.2)^{a}	3.2 (± 0.3)^{b}	2.1 (± 0.2)^{bc}	1.4 (± 0.3)^{c}
β-carotene	Intact *Nannochloropsis*	3.8 (± 0.4)^{a}	2.37 (± 0.05)^{b}	1.38 (± 0.06)^{bc}	1.1 (± 0.3)^{c}
	Disrupted *Nannochloropsis*	3.32 (± 0.03)^{a}	2.4 (± 0.3)^{b}	1.79 (± 0.12) ^{b}	0.95 (± 0.16)^{c}
	Nannochloropsis oil	2.2 (± 0.2)^{a}	2.8 (± 0.2)^{a}	1.21 (± 0.18)^{b}	0.65 (± 0.13)^{b}
	Fish oil	0.99 (± 0.02)^{a}	0.57 (± 0.03)^{b}	0.23 (± 0.02)^{c}	0.16 (± 0.03)^{c}
Zeaxanthin	Intact *Nannochloropsis*	4.00 (± 0.10)^{a}	1.91 (± 0.11)^{b}	1.42 (± 0.09)^{c}	0.92 (± 0.18)^{d}
	Disrupted *Nannochloropsis*	2.88 (± 0.13)^{a}	1.44 (± 0.09)^{b}	1.11 (± 0.06)^{bc}	0.73 (± 0.18)^{c}
	Nannochloropsis oil	1.53 (± 0.08)^{a}	0.69 (± 0.07)^{b}	0.33 (± 0.14)^{c}	0.22 (± 0.05)^{c}
	Fish oil	-	-	-	-
Antheraxanthin	Intact *Nannochloropsis*	0.52 (± 0.05)	-	-	-
	Disrupted *Nannochloropsis*	0.26 (± 0.05)	-	-	-
	Nannochloropsis oil	0.56 (± 0.06)	-	-	-
	Fish oil	-	-	-	-

C	Dosage form	Sterilized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	Intact *Nannochloropsis*	5.1 (± 0.6)[a]	3.1 (± 0.9)[a]	3.59 (± 0.22)[a]	3.3 (± 0.6)[a]
	Disrupted *Nannochloropsis*	4.97 (± 0.02)[a]	2.7 (± 0.4)[b]	2.4 (± 0.5)[b]	1.88 (± 0.14)[b]
	Nannochloropsis oil	5.52 (± 0.05)[a]	4.3 (± 0.6)[a]	2.5 (± 0.2)[b]	1.6 (± 0.2)[b]
	Fish oil	5.38 (± 0.12)[a]	3.2 (± 0.4)[b]	2.1 (± 0.4)[bc]	1.3 (± 0.4)[c]
β-carotene	Intact *Nannochloropsis*	4.0 (± 0.3)[a]	1.7 (± 0.8)[b]	1.8 (± 0.3)[b]	1.5 (± 0.4)[b]
	Disrupted *Nannochloropsis*	3.6 (± 0.5)[a]	1.9 (± 0.2)[b]	1.3 (± 0.2)[b]	0.86 (± 0.08)[b]
	Nannochloropsis oil	2.5 (± 0.3)[a]	2.8 (± 0.2)[a]	2.52 (± 0.16)[a]	0.85 (± 0.19)[b]
	Fish oil	0.92 (± 0.08)[a]	0.57 (± 0.08)[b]	0.24 (± 0.08)[c]	0.15 (± 0.02)[c]
Zeaxanthin	Intact *Nannochloropsis*	3.8 (± 0.2)[a]	0.9 (± 0.4)[b]	0.64 (± 0.12)[b]	0.55 (± 0.17)[b]
	Disrupted *Nannochloropsis*	2.52 (± 0.17)[a]	0.75 (± 0.07)[b]	0.45 (± 0.06)[bc]	0.28 (± 0.03)[c]
	Nannochloropsis oil	1.3 (± 0.11)[a]	0.4 (± 0.19)[b]	0.42 (± 0.09)[b]	0.21 (± 0.06)[b]
	Fish oil	-	-	-	-
Antheraxanthin	Intact *Nannochloropsis*	0.21 (± 0.08)	-	-	-
	Disrupted *Nannochloropsis*	0.08 (± 0.03)	-	-	-
	Nannochloropsis oil	0.13 (± 0.04)	-	-	-
	Fish oil	-	-	-	-

5.4. CONCLUSIONS

The first observed difference between the dosage forms was a lower practicality in handling of *Nannochloropsis* oil. Stickiness to equipment during mixing and high pressure homogenization resulted in large physical losses. Based on this result, the use of *Nannochloropsis* oil as n-3 LC-PUFA source is not recommended from an economic point of view.

The n-3 LC-PUFA derived from all investigated sources did not decrease throughout high pressure homogenization, pasteurization and sterilization when incorporated in a tomato puree. However, during storage, the dosage form showed an impact on the oxidative stability of the enriched purees. Purees supplemented with (intact or disrupted) *Nannochloropsis* biomass performed slightly better compared to *Nannochloropsis* oil and clearly better than fish oil. A tomato puree (full of antioxidants) could not prevent n-3 LC-PUFA rich oil from lipid oxidation.

This higher oxidative stability could primarily be linked to the carotenoids derived from the microalgae. Large reductions in the carotenoids (because of their antioxidant action) were observed during storage, and the carotenoids derived from photoautotrophic microalgae degraded preferably. Most likely, the stronger antioxidant capacity of the zeaxanthin, antheraxanthin and β-carotene compared to lycopene and/or their closer location to the n-3 LC-PUFA (when using photoautotrophic microalgae, carotenoids and n-3 LC-PUFA are located in the same cell or even cell organelle) leads to the higher oxidative stability of tomato purees enriched with photoautotrophic microalgae.

Nannochloropsis as intact and (partially) disrupted biomass were thus the preferred dosage forms of n-3 LC-PUFA in enriching a tomato puree without compromising the oxidative stability.

Chapter 6:

IMPACT OF MICROALGAL SPECIES ON THE OXIDATIVE STABILITY OF N-3 LC-PUFA ENRICHED TOMATO PUREE

This chapter is based on:

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: 1–14.

6.1. INTRODUCTION

In chapter 4 the impact of microalgal species on the oxidative stability of aqueous model systems was investigated. It is however hard to predict if similar conclusions can be drawn for vegetable purees, rich in endogenous antioxidants.

This chapter therefore addresses the impact of microalgal species on the oxidative stability of tomato purees. Biomass of three photoautotrophic microalgae (*Isochrysis sp., Nannochloropsis sp.* and *Phaeodactylum sp.*), with different antioxidant profile and cell wall composition, and one heterotrophic microalga (*Schizochytrium sp.*) was selected. Given the fact that chapter 5 showed that no significant differences in oxidative stability could be observed between tomato purees enriched with (partially) disrupted and non-disrupted biomass, it was decided to only use the non-disrupted biomass. The effect of mechanical (high pressure homogenization) and thermal (pasteurization or sterilization) processing typical for this type of products, was also investigated. To fill the gap in the current knowledge on the n-3 LC-PUFA enrichment of vegetable-based products, tomato puree was selected as food system and was enriched to reach a concentration of 80 mg n-3 LC-PUFA/100 g puree. The biomass itself and all enriched tomato products were analyzed for their amount of n-3 LC-PUFA, antioxidants and free fatty acids. Additionally, the primary and secondary lipid oxidation products were measured to follow the oxidative stability and the carotenoid degradation of the enriched tomato products during a 12 week storage experiment.

6.2. MATERIALS AND METHODS

6.2.1. MICROALGAL SPECIES

Four different n-3 LC-PUFA rich microalgae (3 photoautotrophic and 1 heterotrophic) were used in this chapter. *Isochrysis* sp. and *Nannochloropsis* sp. biomass was obtained from Proviron (Hemiksem,

Belgium), *Schizochytrium* sp. biomass from Bunge (St Just Desvern, Spain) and *Phaeodactylum* sp. biomass from Necton (Olhão, Portugal). All biomass was delivered dry and stored at -80°C until further use.

6.2.2. PREPARATION OF TOMATO PUREE

Tomatoes (cultivar *Bonaparte*) were obtained from Proefcentrum Hoogstraten (Hoogstraten, Belgium). The tomato puree was prepared according to the method described in section 5.2.2.

6.2.3. EXPERIMENTAL SET-UP

Figure 6.1 represents the experimental set-up, based on the *2x2* principle, of this chapter. For each of the different microalgae, a tomato puree was prepared according to the steps shown in Figure 5.2.

6.2.4. PREPARATION AND PROCESSING OF ENRICHED TOMATO PUREE

The different microalgal species (*Isochrysis sp.*, *Nannochloropsis sp., Phaeodactylum sp.* and *Schizochytrium sp.*) were added to the thawed tomato puree in order to reach a concentration of 80 mg n-3 LC-PUFA/100 g puree. The enriched tomato purees (with a pH of 4.38 ± 0.15) were prepared, processed and stored according to the method described in section 5.2.4. Each enriched puree was prepared in duplicate.

6.2.5. ANALYSES

6.2.5.1. LIPID EXTRACTION

The lipids were extracted by a chloroform/methanol (1/1) extraction from the freeze dried puree as described in section 3.2.2.1. This analysis was performed in duplicate on each sample.

6.2.5.2. DETERMINATION OF N-3 LC-PUFA CONTENT

The n-3 LC-PUFA content was determined by chromatographic separation and FID detection after methylation of the extracted lipids according to the method described in section 4.2.3.2. The analysis was performed in duplicate.

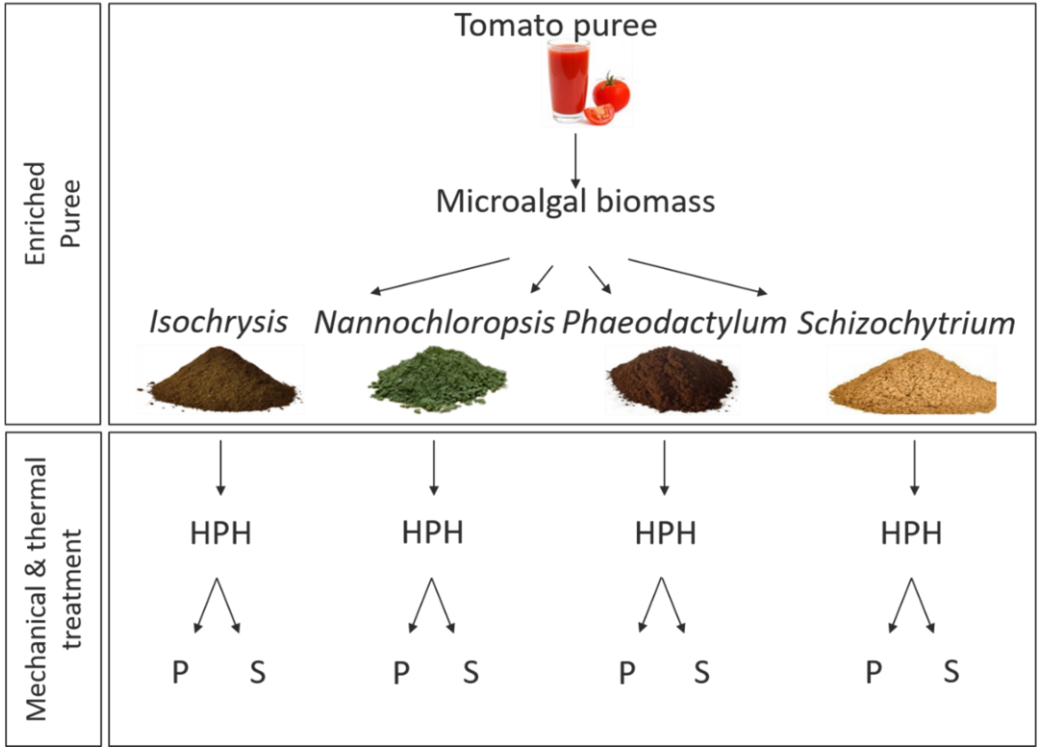


***Figure 6.1**. Experimental set-up of this chapter in which HPH stands for high pressure homogenization, P for pasteurization and S for sterilization. Each system was made in duplicate.*

6.2.5.3. DETERMINATION OF TOTAL FREE FATTY ACID CONTENT

The total free fatty acid content was determined by chromatographic separation after the formation of diethylamide derivatives as described in section 4.2.3.4. The determination of total free fatty acid content was performed in duplicate.

6.2.5.4. DETERMINATION OF CAROTENOIDS

The carotenoid content was determined by HPLC-PAD after extraction with acetone/methanol (7/3) as described in section 5.2.5.4. The analysis was performed in duplicate.

6.2.5.5. DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

The determination of the phenolic content was done with the Folin-Ciocalteu reagent according to the method section 5.2.5.5. The analysis was performed in duplicate.

6.2.5.6. DETERMINATION OF ASCORBIC ACID

The ascorbic acid content was determined by HPLC and fluorescence detection after a derivatization reaction according to the method described in section 5.2.5.6. The analysis was performed in duplicate.

6.2.5.7. DETERMINATION OF TOCOPHEROLS

The amount of tocopherols was determined by HPLC and fluorescence detection according to the method described in section 5.2.5.7. The analysis was performed in duplicate.

6.2.5.8. DETERMINATION OF PRIMARY OXIDATION PRODUCTS

The determination of the primary oxidation products was performed according to the FOX method as described in section 3.2.2.3. The analysis was performed in duplicate. All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

6.2.5.9. DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Volatile secondary oxidation products were analyzed with HS-SPME GC-MS according to the method described in section 3.2.2.7. The semi quantitative concentrations of the identified volatile compounds were calculated as the area of the volatile indicator compound divided by the response factor of the external standard hexanal (spiked in a tomato based suspension). All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

6.2.6. CORRECTION FOR PHYSICAL LOSSES

All parameters were expressed per 100 g puree and corrected for physical losses according to the method described in section 5.2.6.

6.2.7. STATISTICAL ANALYSIS

Comparing the composition of the microalgae was statistically performed by a one way ANOVA for each of the studied variables. Results of the amount of n-3 LC-PUFA, antioxidants and free fatty acids in the enriched tomato purees were statistically evaluated by a two way ANOVA to consider the impact of processing and microalgal species. The oxidative stability (primary and secondary oxidation) was evaluated by a two way ANOVA to consider the impact of time and microalgal species, for each of the processing types. The degradation of carotenoids (for each carotenoid individual) was evaluated by a one way ANOVA to consider the impact of time for each microalgal species - processing intensity combination. Significant differences among enriched purees were determined by a *post hoc* Tukey test. All statistical tests were performed with $\alpha = 0.05$ (JMP Pro 12.1, SAS Institute Inc.).

6.3. RESULTS AND DISCUSSION

6.3.1. CHARACTERIZATION OF MICROALGAL BIOMASS

Each used microalgal biomass contained a different concentration of n-3 LC-PUFA, resulting in a different incorporation concentration in each of the tomato purees in order to reach the fixed concentration of 80 mg n-3 LC-PUFA /100 g tomato puree. The incorporation concentrations of the biomass were 0.26 g biomass/100 g enriched puree for *Schizochytrium*, 2.14 g biomass/100 g enriched puree for *Nannochloropsis*, 3.7 g biomass/100 g enriched puree for *Phaeodactylum* and 6.6 g biomass/100 g enriched puree for *Isochrysis.* This implies that when comparing the enriched tomato purees differences could not only be explained by differences in composition but also by differences in incorporation concentration. To facilitate the correct interpretation, first the composition of the different biomasses is discussed (Table 6.1).

With a concentration of 337.4 mg n-3 LC-PUFA/g biomass, the heterotrophic microalga *Schizochytrium* contained the largest amount of n-3 LC-PUFA. Logically, the incorporation concentration was thus lower compared to the photoautotrophic microalgae. *Nannochloropsis* contained 39.5 mg n-3 LC-PUFA/g biomass, while *Phaeodactylum* and *Isochrysis* only had 25.6 and 14.8 mg n-3 LC-PUFA/g biomass, respectively. In *Nannochloropsis* and *Phaeodactylum* biomass the n-3 LC-PUFA was EPA, while for *Isochrysis* and *Schizochytrium* DHA was present. The observed results were in line with the results of Ryckebosch *et al.* [50] and Morita *et al.* [213].

In terms of free fatty acids, large differences between the used microalgal species were observed. *Isochrysis* contained 72.4 mg free fatty acids/g biomass, which was significantly more than the 40.3, 17.6 and 5.6 mg free fatty acids/g biomass for *Phaeodactylum*, *Nannochloropsis* and *Schizochytrium*, respectively. Balduyck *et al.* [66] showed that free fatty acids are formed during wet storage and the cell (wall) integrity explains the differences between different species. It was therefore not surprising

Table 6.1. *n-3 LC-PUFA, free fatty acid and antioxidant composition of Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium biomass, expressed as mg/g biomass, mean (± SD). For each compound, statistical differences are indicated with a different letter, α=0.05.*

	Isochrysis	***Nannochloropsis***	***Phaeodactylum***	***Schizochytrium***
Incorporation concentration (g/100 g tomato puree)	6.60 (± 0.01) [a]	2.14 (± 0.01) [c]	3.70 (± 0.01) [b]	0.24 (± 0.01) [d]
n-3 LC-PUFA (mg/g biomass)	12.0 (±0.2)[d]	37.3 (±0.9)[b]	21.5 (±0.4)[c]	337.4 (±10.9)[a]
Free fatty acids (mg/g biomass)	72.4 (±0.7)[a]	17.6 (±0.6)[c]	40.3 (±2.9)[b]	5.6 (±0.4)[d]
Carotenoids (mg/g biomass)				
β-carotene	0.78 (±<0.01)[b]	1.42 (±0.05)[a]	0.78 (±<0.01)[b]	-
Zeaxanthin	-	1.42 (±0.06)	-	-
Antheraxanthin	-	0.97 (±0.03)	-	-
Violaxanthin	-	0.62 (±0.05)	-	-
Lutein	-	0.46 (±0.02)	-	-
Fucoxanthin	3.76 (±0.11)[b]	-	7.14 (±0.01)[a]	-
Diatoxanthin	0.47 (±0.02)[b]	-	1.11 (±0.01)[a]	-
Echinenone	0.9 (±0.2)	-	-	-
Chlorophylls (mg/g biomass)				
Chlorophyll a	0.41 (±0.03)[c]	13.2 (±0.3)[a]	6.12 (±0.06)[b]	-
Chlorophyll c2	1.99 (±0.05)[b]	-	2.21 (±<0.01)[a]	-
Phenolic compounds (mg/g biomass)	5.8 (±0.3)[a]	2.94 (±0.02)[b]	5.78 (±0.06)[a]	2.66 (±0.01)[b]
Tocopherols (mg/g biomass)	0.98 (±0.03)[a]	0.61 (±0.17)[ab]	0.22 (±0.01)[bc]	0.04 (±<0.01)[c]
Ascorbic acid (mg/g biomass)	0.30 (±0.01)[b]	1.59 (±0.05)[a]	0.08 (±0.01)[c]	0.38 (±0.04)[b]

that the species with the weaker cell wall, namely *Isochrysis* and *Phaeodactylum*, contained the most free fatty acids.

The observed carotenoid profiles were in line with literature [79,185]. *Nannochloropsis* was characterized by a variety of carotenoids (zeaxanthin (1.42 mg/g biomass), β-carotene (1.42 mg/g biomass), antheraxanthin (0.97 mg/g biomass), violaxanthin (0.62 mg/g biomass), and lutein (0.46 mg/g biomass)), *Isochrysis* and *Phaeodactylum* mainly contained fucoxanthin (3.76 and 7.14 mg/g biomass respectively) and *Schizochytrium* was lacking carotenoids. The three photoautotrophic microalgae also contained chlorophyll in amounts up to 13.2 mg/g biomass in the case of *Nannochloropsis*. The presence of pigments in photoautotrophic microalgae and their absence in heterotrophic microalgae can be attributed to their pathway of energy supply.

Clear differences between the species in content of phenolic compounds, tocopherols and ascorbic acid content were observed. The phenolic compound concentration in *Isochrysis* (5.8 mg/g biomass) and *Phaeodactylum* (5.78 mg/g biomass) was significantly higher compared to *Nannochloropsis* (2.94 mg/g biomass) and *Schizochytrium* (2.66 mg/g biomass). The results were in line with those of Goiris *et al.* [185]. Tocopherols, mainly α-tocopherol, were present in the highest amounts in *Isochrysis* (0.98 mg/g biomass), followed by *Nannochloropsis* (0.61 mg/g biomass), *Phaeodactylum* (0.22 mg/g biomass) and *Schizochytrium* (0.04 mg/g biomass). Significantly higher concentrations of ascorbic acid were found in *Nannochloropsis* (1.59 mg/g biomass), followed by *Schizochytrium* and *Isochrysis* (0.38 and 0.30 mg/g biomass) and *Phaeodactylum* (0.08 mg/g biomass). These results were not entirely in line with those from the literature [199,214], which may be attributed to differences in cultivation or analysis method.

6.3.2. N-3 LC-PUFA CONTENT OF ENRICHED TOMATO PUREE

As a concentration of 80 mg n-3 LC-PUFA/100 g enriched puree was required in the final product, it was important to investigate the impact of processing, including possible species dependence. Figure 6.2 shows

the amount of n-3 LC-PUFA/100 g enriched puree after the correction for physical losses after each processing step. No significant impact of processing could be observed for any of the species used.

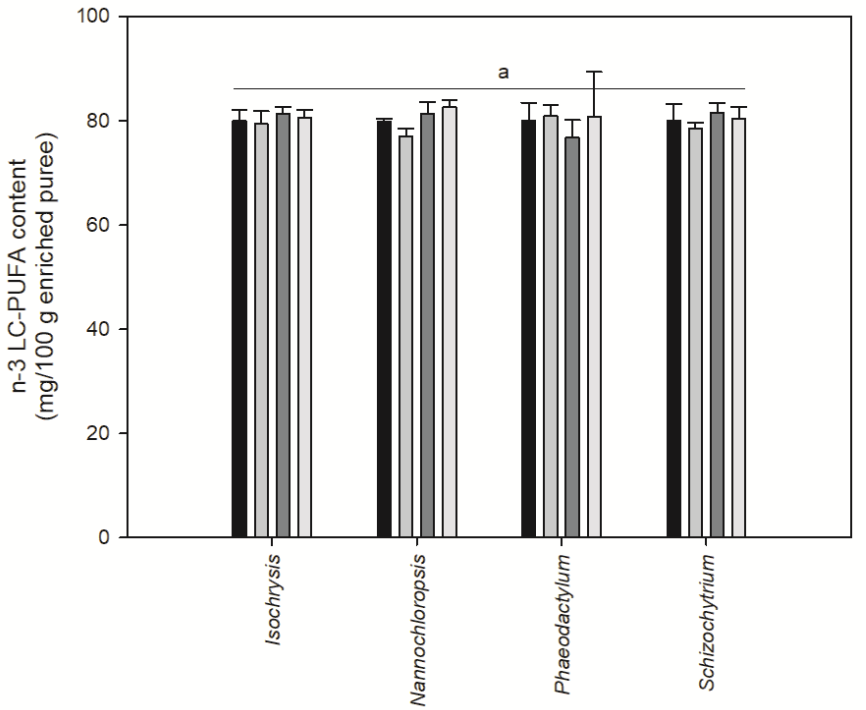


Figure 6.2. *Amount of n-3 LC-PUFA (expressed as mg/100 g enriched puree) after correction for physical losses in the tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium. The amount in the enriched puree was measured after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). No significant differences were observed, α=0.05.*

The absence of the impact of processing was not self-evident. In chapter 4 the impact of high pressure homogenization combined with pasteurization on acidic (pH 4) aqueous model systems supplemented with microalgal biomass was investigated. A decrease of 15% n-3 LC-PUFA due to thermal degradation was observed in all systems, expect for those with a high free fatty acid content where a degradation up to 35% was observed. The contrast with the results obtained in this research, with the same species and the same or even more intensive treatment (in case of

sterilization), could indicate a protective role of the tomato puree, rich in antioxidants. Also, the impact of high amounts of free fatty acids in the system was nullified when a tomato puree was used.

When n-3 LC-PUFA rich microalgae were incorporated in food products only a limited amount of studies investigated the impact of processing on n-3 LC-PUFA content. In the studies of Gouveia *et al.* [76] and Fradique *et al.* [77], no significant impact of cooking on n-3 LC-PUFA derived from the photoautotrophic microalga *Diacronema* sp. was reported. Lee *et al.* [94] observed a reduction in the amount of n-3 LC-PUFA when heterotrophic *Crypthecodinium* sp. oil was incorporated in cooked meat products. These authors suggested physical losses as the main explanation for the observed reduction. In this research however, values were corrected for physical losses (section 6.2.6) and as such the results obtained in this chapter are in line with the limited results of previously published research.

6.3.3. OXIDATIVE STABILITY OF ENRICHED TOMATO PUREE

6.3.3.1. LIPID OXIDATION

The formation of primary and secondary oxidation products during 12 weeks of storage at 37°C is shown in Figure 6.3. It was observed that tomato purees supplemented with *Schizochytrium* showed a different pattern compared to the other enriched purees. For each of the treatments the same observation was made. For tomato purees supplemented with *Schizochytrium* the largest amounts of primary oxidation products were measured after 4 weeks of storage. After that, a decrease in primary oxidation products was observed due to the transformation into secondary oxidation products. The resulting levels of the secondary oxidation products confirmed the evolution of the primary oxidation. The amount of volatile compounds had already increased strongly after 4 weeks of storage. Until 12 weeks of storage, the values kept on fluctuating around this level. These observations suggest that pronounced oxidation of the tomato puree enriched with *Schizochytrium*

took place during the first 4 weeks of storage and that very likely the maximum in the primary oxidation was located between week zero and 4.

Tomato purees supplemented with *Isochrysis, Nannochloropsis* and *Phaeodactylum* showed a similar yet distinct evolution compared to those supplemented with *Schizochytrium*. During storage, a slight increase in the primary oxidation products was observed, for each of the treatments. The increase in primary oxidation products of purees supplemented with *Isochrysis* (0.06 meq hydroperoxides/100 g enriched puree) seemed higher than those supplemented with *Nannochloropsis* and *Phaeodactylum* (0.03 meq hydroperoxides/100 g enriched puree). However, for all three photoautotrophic microalgae a limited increase in volatile secondary oxidation products was noticed but no differences between the different microalgae were observable. A statistical analysis of the effect of storage time on the primary and secondary oxidation products showed that none of these increases could be considered as significant. At most, for each of the tomato purees supplemented with photoautotrophic microalgae an indication of the initiation of oxidation could be observed. Based on the results of the primary oxidation this initiation seemed slightly more pronounced in the case of *Isochrysis*.

A reduction in the secondary oxidation compounds between week 0 and week 4 was observed in high pressure homogenized puree supplemented with *Nannochloropsis*. This could be explained by the presence of volatile organic compounds formed *in vivo* in *Nannochloropsis* which were still present at week 0 and decomposed during storage or thermal processing [201,202]. This reduction between week 0 and 4 was also observed in chapter 5.

The observed differences between the oxidative stability of the purees supplemented with photoautotrophic and heterotrophic microalgae were in line with the study of chapter 4. Acidic aqueous model systems (pH 4) supplemented with photoautotrophic microalgal biomass (*Isochrysis*, *Nannochloropsis*, *Phaeodactylum* and *Porphyridium*) showed a higher oxidative stability compared to systems supplemented with *Schizochytrium*. In chapter 4 a decrease in the peroxide value of systems

supplemented with *Schizochytrium* could already be observed from week zero, which means that the oxidation was more pronounced in the model systems. This indicated that the tomato puree helped to improve the oxidative stability, but this was still not sufficient to maintain the oxidative stability in this chapter as already from week 4 on a decrease in the peroxide value was observed. Other studies on the oxidative stability of food products supplemented with oil from *Schizochytrium* showed varying results. Even when preventive actions were taken such as vacuum storage and addition of antioxidants, it was in general not possible to keep the enriched products oxidatively stable [105–108].

The study of Babuskin *et al.* [83] and chapter 5 investigated the oxidative stability of photoautotrophic microalgae in chikkis and a tomato puree, respectively. Babuskin *et al.* [83] stored chikkis supplemented with 3% *Isochrysis* or *Nannochloropsis* for 60 days at 27°C and observed a slight increase in the peroxide value comparable to that in the present chapter. The results of chapter 5 also showed a good oxidative stability of tomato purees enriched with *Nannochloropsis* biomass.

Based on the results of the storage experiment the microalgal species had a clear impact on the oxidative stability. Tomato purees supplemented with photoautotrophic microalgal biomass showed a much better oxidative stability compared to tomato purees supplemented with heterotrophic microalgal biomass. Processing did not have a significant impact on the oxidative stability of the enriched tomato purees. In section 6.3.3.2, the possible causative factors for differences in the oxidative stability are discussed. Their relation to the observed oxidative stability is discussed in section 6.3.3.3.

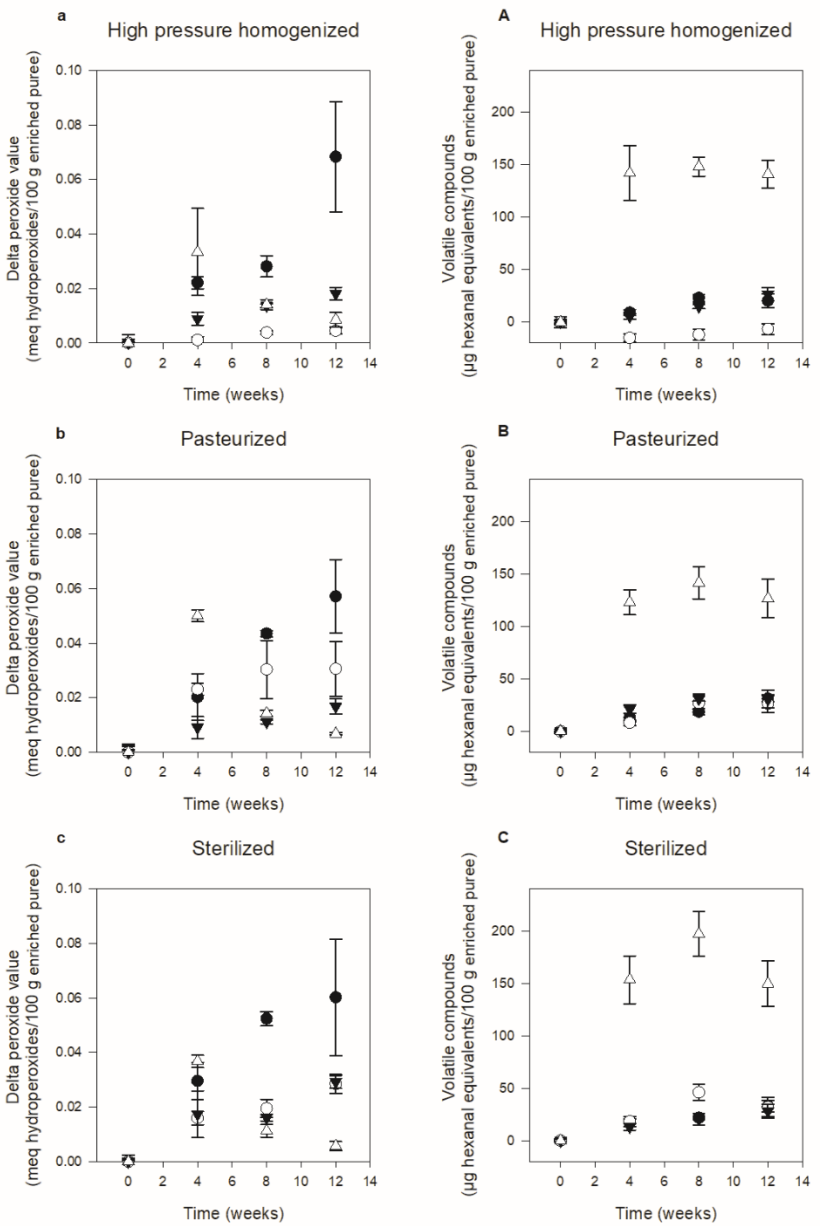


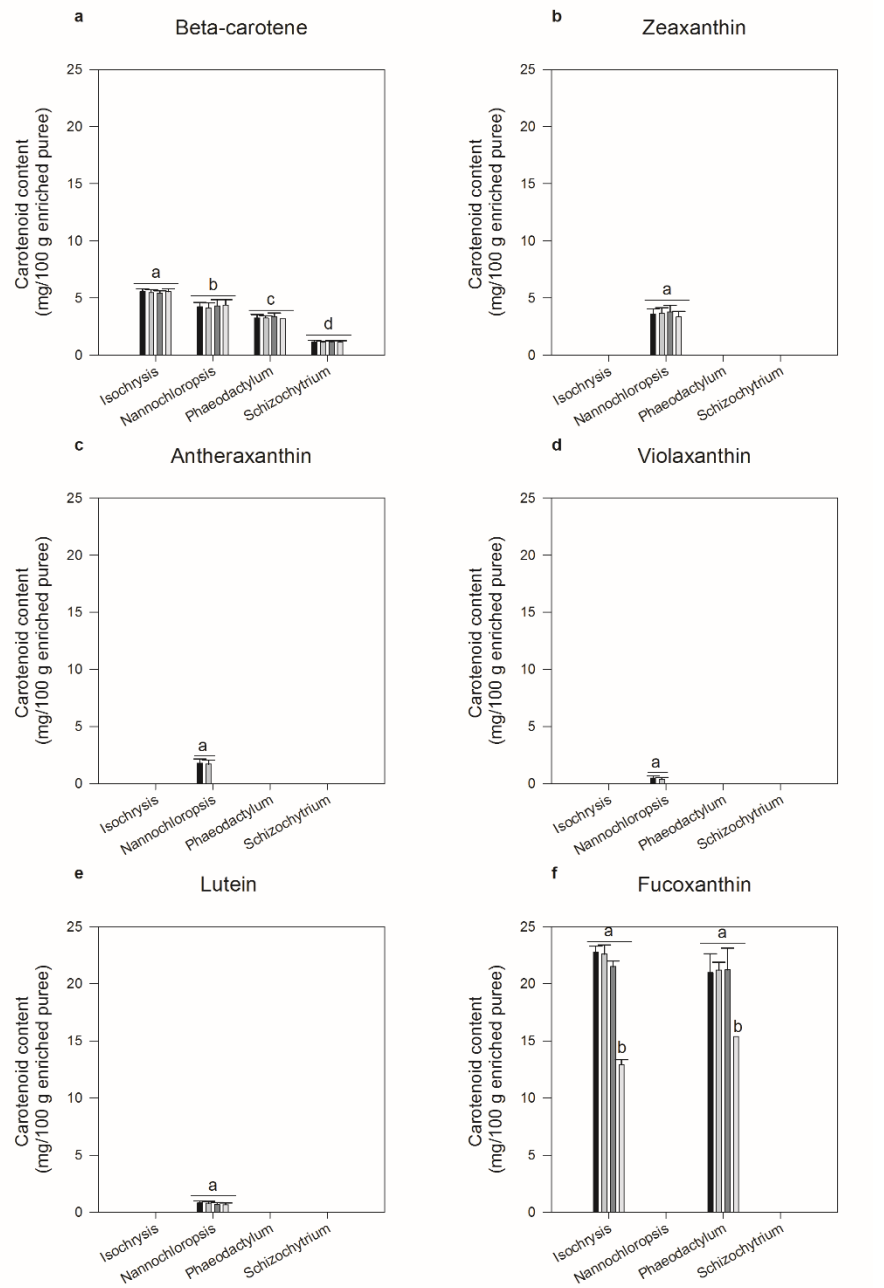
Figure 6.3. *Evolution of primary (small letter) and secondary (capital letter) oxidation products during 12 weeks of storage at 37°C for the tomato purees supplemented with Isochrysis (●), Nannochloropsis (○), Phaeodactylum (▼) and Schizochytrium (Δ) after high pressure homogenization (a & A), pasteurization (b & B) and sterilization (c & C), mean (± SD). The results are expressed as delta values, which is the relative increase or decrease compared to week zero.*

6.3.3.2. INFLUENCING FACTORS

i. *CAROTENOIDS*

Figure 6.4 presents the main carotenoids in the different enriched purees. Lycopene (4.65 mg/100 g enriched puree) originated only from the tomato puree itself, and was therefore independent of the microalgal species (results not shown). The tomato puree as well as the microalgae contained β-carotene. A significant impact of microalgal species on the total amount of β-carotene was therefore observed (Figure 6.4a). A final concentration of β-carotene of 5.5; 4.3; 3.4 and 1.1 mg/100 g enriched puree was obtained for purees supplemented with *Isochrysis, Nannochloropsis, Phaeodactylum* and *Schizochytrium* respectively. The tomato puree itself accounted for 1.1 mg β-carotene/100 g enriched puree, indicating that only the photoautotrophic microalgae provided an extra amount of β-carotene. *Nannochloropsis* biomass contained twice as much β-carotene compared to *Isochrysis* and *Phaeodactylum*, but the higher incorporation concentration of the latter two compensated the lower concentration of β-carotene in the biomass (Table 6.1 and 6.2).

Besides lycopene and β-carotene, purees supplemented with *Nannochloropsis* also contained zeaxanthin (Figure 6.4b), antheraxanthin (Figure 6.4c), lutein (Figure 6.4d) and violaxanthin (Figure 6.4e), with respective amounts of 3.6; 1.8; 0.8 and 0.5 mg/100 g enriched puree. Taking the incorporation concentration and the initial amount in the biomass into account, a higher violaxanthin concentration (1.3 mg/100 g enriched puree) and a lower zeaxanthin concentration (3 mg/100 g enriched puree) was expected. Violaxanthin, antheraxanthin and zeaxanthin can however be converted into each other via the violaxanthin cycle [215]. This process is favored in the presence of ascorbic acid and a pH lower than 6.2 [215], conditions that were fulfilled in the enriched tomato puree.


a
Beta-carotene
Carotenoid content (mg/100 g enriched puree)
25
20
15
10
5
0
a
b
c
d
Isochrysis
Nannochloropsis
Phaeodactylum
Schizochytrium
b
Zeaxanthin
a
c
Antheraxanthin
a
d
Violaxanthin
a
e
Lutein
a
f
Fucoxanthin
a
b
a
b

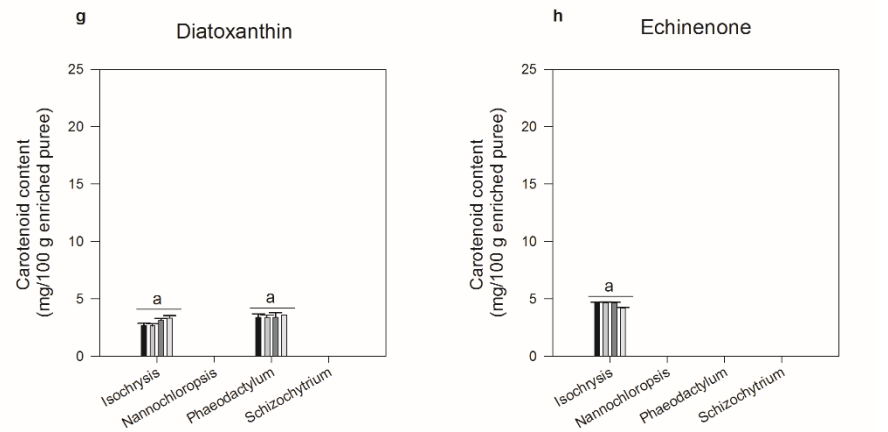


***Figure 6.4**. Carotenoid composition (beta-carotene (a), zeaxanthin (b), antheraxanthin (c), violaxanthin (d), lutein (e), fucoxanthin (f), diatoxanthin (g) and echinenone (h)), for tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences (per carotenoid) are indicated with a different letter, α=0.05.*

Purees supplemented with *Isochrysis* and *Phaeodactylum* contained, besides lycopene and β-carotene, fucoxanthin (Figure 6.4f) and diatoxanthin (Figure 6.4g). No significant differences in the amount of fucoxanthin (23 mg/100 g enriched puree) and diatoxanthin (3 mg/100 g enriched puree) between the purees enriched with *Isochrysis* and *Phaeodactylum* were observed. Although *Phaeodactylum* biomass contained larger amounts of both carotenoids, the higher incorporation concentration of *Isochrysis* compensated for this difference. Echinenone was also present in purees supplemented with *Isochrysis* in a concentration of 4.2 mg/100 g enriched puree (Figure 6.4h).

No impact of processing was observed for lycopene, β-carotene, zeaxanthin, lutein, diatoxanthin and echinenone, while antheraxanthin, violaxanthin and fucoxanthin decreased significantly by thermal processing. Antheraxanthin and violaxanthin were absent in pasteurized and sterilized purees, while fucoxanthin was 40% decreased by sterilization. The observed processing induced degradations were in accordance to literature [204,216]. Hadjal *et al.* [216] investigated the thermal degradation of xanthophylls in acidic model systems and observed that

antheraxanthin and violaxanthin were more sensitive to degradation compared to zeaxanthin and lutein. The presence of one or more epoxy groups was the predominant factor in reducing the thermal stability. Fucoxanthin also contains an epoxy group which makes this carotenoid sensitive to degradation [217].

ii. *TOTAL PHENOLIC COMPOUNDS*

The amount of phenolic compounds is shown in Figure 6.5. Large differences in the amount of phenolic compounds could be observed between tomato purees supplemented with different microalgae. Tomato puree itself supplied approximately 15 mg GAE/100 g tomato puree. Tomato purees supplemented with *Isochrysis* contained the highest amount of phenolic compounds (60 mg GAE/100 g enriched puree), while purees supplemented with *Phaeodactylum* contained 30 mg GAE/100 g enriched puree, a difference that could mainly be attributed to the higher incorporation concentration of *Isochrysis*. Tomato puree supplemented with *Nannochloropsis* had 20 mg GAE/100 g enriched puree and that supplemented with *Schizochytrium* contained 15 mg GAE/100 g enriched puree. The three and four times lower concentration in purees supplemented with *Nannochloropsis* and *Schizochytrium* compared to those supplemented with *Isochrysis* could be explained by a combined effect of the lower concentration in the microalgal biomass itself and the lower incorporation concentration (Table 6.1). No significant impact of processing on the amount of phenolic compounds could be observed. In literature contradictory results were found: either increasing or decreasing amounts, or no significant effects were observed depending on the study [206,218,219]. The processing steps in this research were most similar to those of Dewanto *et al.* [206] who did not observe any significant effect of thermal treatment (30 min at 80°C) on the amount of phenolic compounds in tomato puree.

Table 6.2. *Theoretical calculated values of n-3 LC-PUFA, free fatty acids and antioxidants (mg/100 g enriched puree) of tomato puree enriched with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium, mean (± SD).*

	Isochrysis	*Nannochloropsis*	*Phaeodactylum*	*Schizochytrium*
n-3 LC-PUFA (mg/100 g enriched puree)	79.2 (± 1.3)	79.8 (± 1.9)	79.6 (± 1.5)	81 (± 3)
Free fatty acid (mg/100 g enriched puree)	478 (± 5)	37.7 (± 1.3)	149 (± 11)	1.34 (± 0.10)
Carotenoids (mg/100 g enriched puree)				
β-carotene	6.15 (± 0.08)	4.04 (± 0.14)	3.89 (± 0.05)	1.00 (± <0.01)
Zeaxanthin	-	3.04 (± 0.13)	-	-
Antheraxanthin	-	2.08 (± 0.06)	-	-
Violaxanthin	-	1.33 (± 0.11)	-	-
Lutein	-	0.98 (± 0.11)	-	-
Fucoxanthin	24.8 (± 0.7)	-	26.42 (± 0.04)	-
Diatoxanthin	3.10 (± 0.13)	-	4.11 (± 0.04)	-
Echinenone	6.3 (± 1.4)	-	-	-
Lycopene	4.65 (± 0.02)	4.65 (± 0.02)	4.65 (± 0.02)	4.65 (± 0.02)
Phenolic compounds (mg/100 g enriched puree)	51.1 (± 3.0)	19.13 (± 0.13)	34.2 (± 0.4)	13.48 (± 0.05)
Tocopherols (mg/100 g enriched puree)	7.0 (± 0.2)	1.9 (± 0.5)	1.38 (± 0.06)	0.58 (± 0.14)
Ascorbic Acid (mg/100 g enriched puree)	21.0 (± 0.7)	22.4 (± 0.7)	19 (± 2)	19 (± 2)

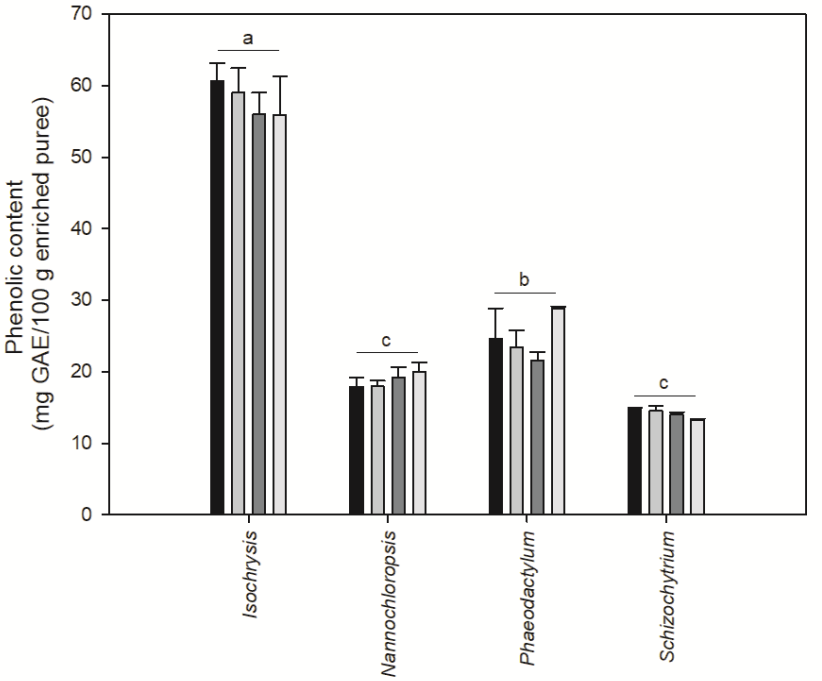


Figure 6.5. *Phenolic content for tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

iii. *TOCOPHEROLS*

Figure 6.6 shows the amounts of tocopherols, mainly α-tocopherol, in the enriched tomato purees.

Tomato puree itself contained 0.66 mg tocopherols/100 g. A clear impact of microalgal species could be observed. Purees supplemented with *Isochrysis* showed the highest amount of tocopherols (6 mg/100 g enriched puree), which could be explained by the higher incorporation concentration of *Isochrysis* biomass and the higher tocopherol concentration in the *Isochrysis* biomass (Table 6.1). Tomato purees supplemented with *Nannochloropsis* contained 2 mg tocopherols/100 g enriched puree, which was significantly higher than purees supplemented with *Phaeodactylum* (1.3 mg/100 g enriched puree). Although the added

amount of *Phaeodactylum* was almost twice as high as that of *Nannochloropsis*, the tocopherol concentration in the *Nannochloropsis* biomass was three times higher. Purees supplemented with *Schizochytrium* had the lowest amount of tocopherols (0.68 mg/100 g enriched puree) which originated mainly from the tomato puree itself. No significant impact of processing was observed on the level of tocopherols which is in line with the research of Seybold *et al.* [220] who showed a high thermal stability of tocopherols.

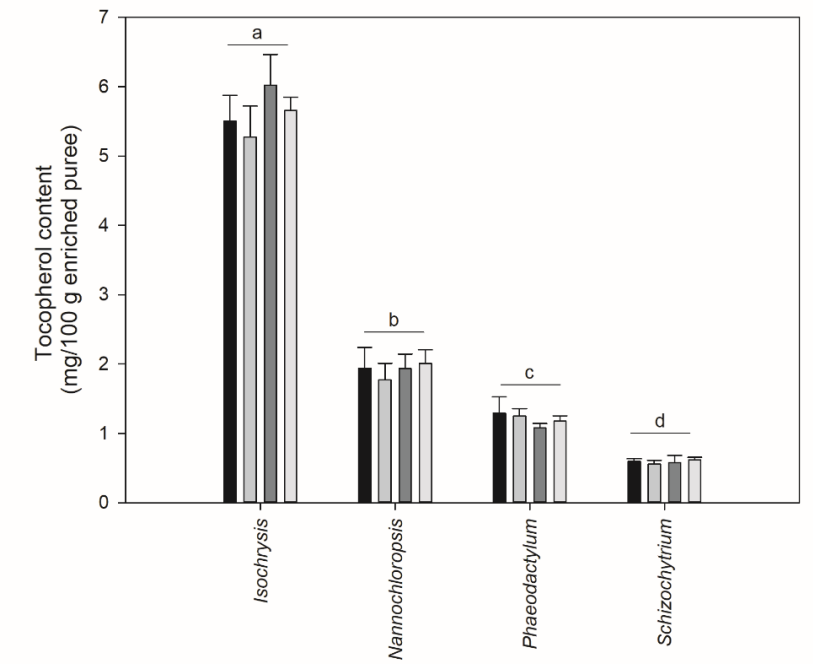


Figure 6.6. *Tocopherol content for tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences are indicated with a different letter, α=0.05.*

iv. *ASCORBIC ACID*

The amount of ascorbic acid in the different enriched purees is shown in Figure 6.7. A similar impact of processing was observed in the purees supplemented with *Nannochloropsis, Phaeodactylum* and *Schizochytrium*. Pasteurization and sterilization reduced the amount of

ascorbic acid drastically, which was expected as ascorbic acid is known as a thermolabile compound [219,221]. Differences in ascorbic acid concentration between the microalgal species (Table 6.1) were negligible due to the high ascorbic acid concentration of the tomato puree (Table 6.2).

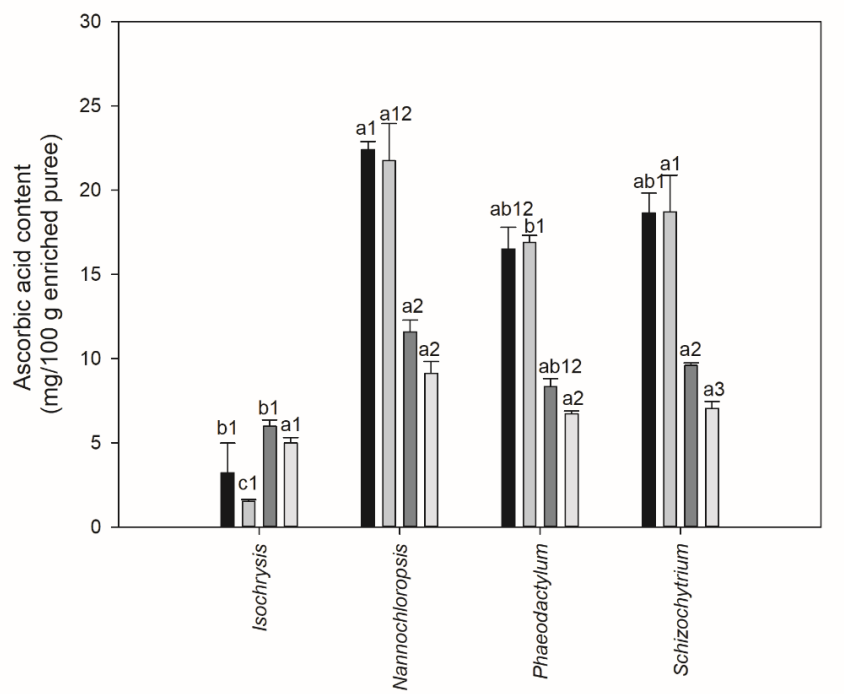


***Figure 6.7**. Ascorbic acid content for tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences between microalgal species within a treatment are indicated with a different letter and statistical differences between treatments within a microalgal species are indicated with a different number, α=0.05.*

The impact of processing for the purees supplemented with *Isochrysis* was different. Although the impact of processing was not significant, the amount of ascorbic acid seemed to increase by thermal processing. Moreover, the amount of ascorbic acid was lower in purees supplemented with *Isochrysis* compared to purees supplemented with *Nannochloropsis, Phaeodactylum* and *Schizochytrium* despite the fact that ascorbic acid was mainly derived from the tomato puree. Based on

the amount of ascorbic acid in *Isochrysis* biomass (Table 6.1) and in tomato puree an ascorbic acid concentration of 21.0 mg/100 g enriched puree was expected after mixing (Table 6.2), which is in the range of the amount observed in purees enriched with *Nannochloropsis, Phaeodactylum* and *Schizochytrium*. The increasing trend after thermal processing suggests an enzymatic degradation of ascorbic acid during sample preparation (by enzymes originating from the microalga *Isochrysis*) in the mixed and high pressure homogenized purees, which was eliminated by denaturation of the enzymes after thermal processing. To confirm this hypothesis an aqueous suspension enriched with ascorbic acid and with and without *Isochrysis* was stored for 12 and 24 hours at room temperature (detailed results not shown). The aqueous suspension enriched with *Isochrysis* after 12 hours only contained 55% of the ascorbic acid content compared to the aqueous suspension enriched with ascorbic acid without *Isochrysis.* Longer storage of the aqueous suspension enriched with *Isochrysis* further reduced, although slightly, the amount of ascorbic acid. This seemed to confirm the hypothesis that *Isochrysis* biomass, probably by the presence of enzymes (e.g. ascorbic acid oxidase), accelerated the degradation of ascorbic acid.

v. *FREE FATTY ACIDS*

The amount of free fatty acids is shown in Figure 6.8. The different processing steps did not influence the amount of free fatty acids in the enriched purees significantly, while the microalgal species had a significant impact. Tomato puree supplemented with *Isochrysis* contained high amounts of free fatty acids (±450 mg/100 g enriched puree). Tomato puree supplemented with *Phaeodactylum* (±150 mg/100 g enriched puree), *Nannochloropsis* (±50 mg/100 g enriched puree) and *Schizochytrium* (±7 mg/100 g enriched puree) had 3, 9 and 64 times less free fatty acids compared to purees supplemented with *Isochrysis,* respectively. This observation could be expected based on the free fatty acid content of the microalgal biomass itself (Table 6.1) and the effect was further enhanced by the differences in incorporation concentration

(Table 6.2). Free fatty acids can cause a rancid smell and should thus be avoided [114].

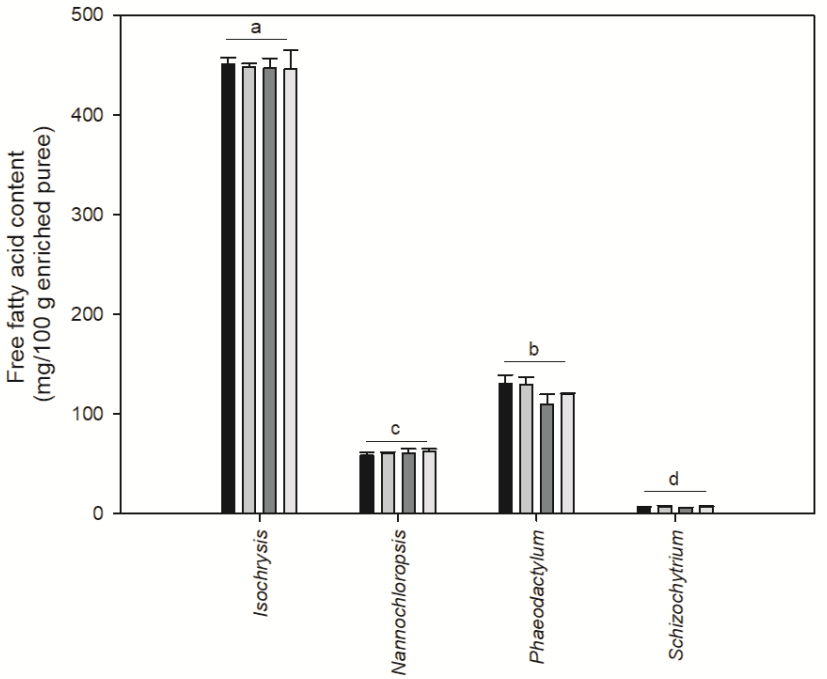


Figure 6.8. *Total free fatty acid content for tomato purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). Statistical differences (per carotenoid) are indicated with a different letter, α=0.05.*

6.3.3.3. RELATION BETWEEN OXIDATIVE STABILITY AND INFLUENCING FACTORS

Large differences in oxidative stability between the purees supplemented with different photoautotrophic and with heterotrophic microalgae were observed (6.3.3.1). In parallel, differences in factors known for their impact on the oxidative stability were seen (6.3.3.2).

Carotenoids have antioxidant properties. Their free radical scavenging capacity is possibly their most important antioxidant mode of action in the prevention of oxidation of the enriched purees, as oxidation in this chapter occurred under dark conditions at only 37°C. Carotenoids differ in antioxidant capacity based on their chemical structure, but a mix of

carotenoids may also lead to a higher antioxidant capacity than the individual carotenoids due to synergistic effects [130]. The antioxidant capacity of lycopene and β-carotene derived from the tomato puree seemed insufficient, as they were not able to keep the purees supplemented with *Schizochytrium* oxidatively stable. Possibly, their scavenging capacity was too low, their concentration in the enriched puree too low or they could not reach the n-3 LC-PUFA in the lipid bodies of the *Schizochytrium* cells. Purees supplemented with photoautotrophic microalgae contained additional carotenoids. Zeaxanthin, antheraxanthin, violaxanthin and extra β-carotene from *Nannochloropsis* and fucoxanthin, diatoxanthin and extra β-carotene from *Isochrysis* and *Phaeodactylum*. *Isochrysis* additionally contained small amounts of echinenone, although its role as an antioxidant seemed to be limited. Without echinenone, the carotenoid composition of purees supplemented with *Isochrysis* was comparable with the carotenoid profile of purees supplemented with *Phaeodactylum*, which were also oxidatively stable. As all purees enriched with photoautotrophic microalgae were oxidatively stable, the presence of extra xanthophylls and/or the presence of extra β-carotene seemed to be important for the oxidative stability but the specific xanthophyll profile (zeaxanthin, antheraxanthin, violaxanthin, fucoxanthin or diatoxanthin) seems of minor importance.

On the other hand, the role of tocopherols, ascorbic acid, phenolic compounds and free fatty acids on the oxidative stability appears to be minor. Phenolic compounds can have an antioxidant capacity. By comparison of Figure 6.3 and Figure 6.5, phenolic compounds however did not seem determinative for the oxidative stability of the enriched tomato purees. Purees supplemented with *Schizochytrium* and *Nannochloropsis* contained comparable amounts of phenolic compounds, while large differences in oxidative stability were observed. Goiris *et al.* [185] showed based on the trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) assays, that phenolic compounds were determinative for the antioxidant capacity of microalgae. Possibly the lower pH of these tomato purees (pH 4.4) can

explain the lower antioxidant activity of the phenolic compounds [114]. Incorporation of photoautotrophic microalgae resulted in a higher tocopherol concentration, but the differences between purees supplemented with *Nannochloropsis* and *Phaeodactylum* and purees supplemented with *Schizochytrium* were, in contrast to the differences in oxidative stability, limited. Moreover, purees supplemented with *Isochrysis*, which had visually the highest intention to oxidation of the purees supplemented with photoautotrophic microalgae, contained the highest tocopherol concentration. Safafar *et al.* [222] also showed, based on the DPPH, FRAP and TEAC assay, a limited contribution of tocopherols to the antioxidant capacity of microalgae. Ascorbic acid was highly influenced by processing, while the oxidative stability was not, leading to the conclusion that ascorbic acid is not determinative. The high amounts of free fatty acids in purees supplemented with *Isochrysis* can possibly explain the larger initiation to oxidation compared to the purees supplemented with other photoautotrophic microalgae. But in general the impact of free fatty acids is supposedly minor as the purees with the lowest amount of free fatty acids (*Schizochytrium*) showed the highest degree of oxidation.

Based on above, it was thus hypothesized that the higher oxidative stability of tomato purees supplemented with photoautotrophic microalgae could primarily be attributed to the presence of endogenous carotenoids in the photoautotrophic microalgal biomass. In order to support this hypothesis the evolution of the carotenoid content during the 12 weeks of storage was investigated. Tables 6.3a−c show the evolution of carotenoids originating from the microalgae (zeaxanthin, antheraxanthin, fucoxanthin, diatoxanthin and part of the β-carotene) and the evolution of those derived from tomato puree (lycopene and part of the β-carotene). For clarity, these tables only show the results of the main carotenoids (> 1 mg/100 g enriched puree at week zero in at least one of the enriched purees).

Zeaxanthin and antheraxanthin (*Nannochloropsis*), fucoxanthin and diatoxanthin (*Isochrysis* and *Phaeodactylum*) and β-carotene (partially

derived from *Nannochloropsis, Isochrysis* and *Phaeodactylum* and partially from tomato puree) showed, irrespective of processing, a significant reduction from week 4. Additionally, these reductions were drastic with a loss of 80, 82, 85, 100 and 100% during the 12 weeks of storage, respectively for diatoxanthin, β-carotene, zeaxanthin, fucoxanthin and antheraxanthin.

Notably, lycopene derived from the tomato puree, only showed a significant decrease in the later stages of the storage period. Moreover, this degradation was much lower (up to 45%) compared to the reductions observed in the carotenoids derived from the microalgae itself. On the other hand, the degradation of lycopene in tomato puree enriched with *Schizochytrium* was larger (up to 65%) and occurred earlier in the storage period. This suggests that if no microalgal carotenoids are present in the enriched puree, the carotenoids from the tomato puree degrade more intensively. Although a degradation in lycopene and β-carotene was observed in the purees enriched with *Schizochytrium*, this was insufficient to maintain their oxidative stability.

The degradation of carotenoids during storage on the one hand and the fact that if carotenoids derived from photoautotrophic microalgae were present, they were preferably degraded to act as antioxidant on the other hand confirmed the hypothesis that primarily the carotenoids of microalgae improved the oxidative stability. Further research should investigate if the type of antioxidant (xanthophylls and/or β-carotene versus lycopene), the location of the carotenoids (when using photoautotrophic microalgae, carotenoids and n-3 LC-PUFA are located in the same cell or even cell organelle) or a combined effect, could explain this observation.

Table 6.3. *Evolution of the main carotenoids (> 1 mg/100 g enriched puree) of purees supplemented with Isochrysis, Nannochloropsis, Phaeodactylum and Schizochytrium after high pressure homogenization (a), pasteurization (b) and sterilization (c) during 12 weeks of storage, mean (± SD). Statistical differences in function of time (per enriched puree) are indicated with a different letter, α=0.05.*

a	Microalgal species	High pressure homogenized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	*Isochrysis*	4.0 (± 0.3)^{a}	3.3 (± 0.3)^{a}	3.4 (± 0.3)^{a}	3.0 (± 0.2)^{a}
	Nannochloropsis	4.58 (± 0.09)^{a}	4.71 (± 0.12)^{a}	4.33 (± 0.17)^{a}	3.3 (± 0.03)^{b}
	Phaeodactylum	4.39 (± 0.17)^{a}	3.53 (± 0.11)^{b}	3.0 (± 0.2)^{bc}	2.6 (± 0.3)^{c}
	Schizochytrium	4.6 (± 0.3)^{a}	3.3 (± 0.6)^{ab}	2.6 (±0.4)^{b}	2.31 (± 0.16)^{b}
β-carotene	*Isochrysis*	5.5 (± 0.2)^{a}	3.4 (± 0.3)^{b}	3.2 (± 0.3)^{b}	2.7 (± 0.3)^{b}
	Nannochloropsis	4.1 (± 0.4)^{a}	2.9 (± 0.3)^{b}	1.8 (± 0.2)^{c}	1.6 (± 0.2)^{c}
	Phaeodactylum	3.4 (± 0.3)^{a}	1.98 (± 0.09)^{b}	1.57 (± 0.04)^{bc}	1.14 (± 0.13)^{c}
	Schizochytrium	1.13 (± 0.04)^{a}	0.5 (± 0.2)^{b}	0.31 (± 0.06)^{b}	0.27 (± 0.03)^{b}
Zeaxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	3.6 (± 0.4)^{a}	2.5 (± 0.4)^{b}	1.4 (± 0.07)^{bc}	0.9 (±0.2)^{c}
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-
Antheraxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	1.7 (± 0.3)	-	-	-
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-

a	Microalgal species	Week 0	Week 4	Week 8	Week 12
Fucoxanthin	*Isochrysis*	22.6 (± 0.6)^{a}	1.84 (± 0.03)^{b}	-	-
	Nannochloropsis	-	-	-	-
	Phaeodactylum	23 (± 2)^{a}	2.4 (± 0.4)^{b}	0.42 (± 0.12)^{c}	-
	Schizochytrium	-	-	-	-
Diatoxanthin	*Isochrysis*	2.64 (± 0.11)^{a}	1.18 (± 0.14)^{b}	0.89 (± 0.08)^{bc}	0.64 (± 0.07)^{c}
	Nannochloropsis	-	-	-	-
	Phaeodactylum	3.6 (± 0.3)^{a}	1.83 (± 0.03)^{b}	1.27 (± 0.04)^{bc}	0.74 (± 0.18)^{c}
	Schizochytrium	-	-	-	-

b	Microalgal species	Pasteurized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	*Isochrysis*	4.3 (± 0.3)^{a}	3.9 (± 0.3)^{ab}	3.7 (± 0.2)^{ab}	3.1 (± 0.2)^{b}
	Nannochloropsis	4.70 (± 0.03)^{a}	4.0 (± 0.3)^{ab}	3.9 (± 0.8)^{ab}	2.6 (± 0.3)^{b}
	Phaeodactylum	4.5 (± 0.2)^{a}	3.3 (± 0.2)^{b}	3.1 (± 0.7)^{b}	3.1 (± 0.2)^{b}
	Schizochytrium	4.9 (± 0.2)^{a}	2.94 (± 0.17)^{b}	2.14 (± 0.06)^{bc}	1.7 (± 0.5)^{c}
β-carotene	*Isochrysis*	5.42 (± 0.04)^{a}	3.5 (± 0.2)^{b}	2.39 (± 0.17)^{bc}	1.6 (± 0.6)^{c}
	Nannochloropsis	4.3 (± 0.4)^{a}	2.0 (± 0.2)^{b}	1.2 (± 0.3) ^{bc}	0.8 (± 0.2)^{c}
	Phaeodactylum	3.4 (± 0.2)^{a}	2.0 (± 0.2)^{b}	2.0 (± 0.2)^{b}	1.8 (± 0.2)^{b}
	Schizochytrium	1.15 (± 0.04)^{a}	0.43 (± 0.02)^{b}	0.26 (± 0.02)^{c}	0.21 (± 0.06)^{c}

b	Microalgal species	Week 0	Week 4	Week 8	Week 12
Zeaxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	3.8 (± 0.4)[a]	2.04 (± 0.08)[b]	1.27 (± 0.08)[bc]	0.56 (± 0.08)[c]
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-
Antheraxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	-	-	-	-
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-
Fucoxanthin	*Isochrysis*	21.4 (± 0.3)[a]	4.85 (± 0.12)[b]	0.90 (± 0.02)[c]	-
	Nannochloropsis	-	-	-	-
	Phaeodactylum	21.2 (± 1.8)[a]	8.9 (± 0.6)[b]	5.7 (± 0.9)[bc]	1.7 (± 0.2)[c]
	Schizochytrium	-	-	-	-
Diatoxanthin	*Isochrysis*	3.12 (± 0.07)[a]	1.96 (± 0.02)[b]	1.21 (± 0.07)[c]	0.8 (± 0.2)[c]
	Nannochloropsis	-	-	-	-
	Phaeodactylum	3.4 (± 0.3)[a]	2.09 (± 0.06)[b]	2.24 (± 0.12)[b]	1.79 (± 0.07)[b]
	Schizochytrium	-	-	-	-

c	Microalgal species	Sterilized			
		Week 0	Week 4	Week 8	Week 12
Lycopene	*Isochrysis*	4.0 (± 0.3)[a]	3.6 (± 0.2)[ab]	3.60 (± 0.07)[ab]	3.0 (± 0.3)[b]
	Nannochloropsis	4.91 (± 0.16)[a]	3.9 (± 0.3)[a]	4.0 (± 0.7)[a]	3.1 (± 0.9)[a]
	Phaeodactylum	4.38 (± 0.18)[a]	3.25 (± 0.12)[b]	3.10 (± 0.02)[b]	3.15 (± 0.05)[b]
	Schizochytrium	4.95 (± 0.09)[a]	2.85 (± 0.09)[b]	2.5 (± 0.3)[b]	2.4 (± 0.7)[b]
β-carotene	*Isochrysis*	5.55 (± 0.06)[a]	3.08 (± 0.10)[b]	2.58 (± 0.08)[bc]	2.1 (± 0.3)[c]
	Nannochloropsis	4.4 (± 0.3)[a]	2.24 (± 0.08)[b]	1.7 (± 0.6)[b]	1.2 (± 0.4)[b]
	Phaeodactylum	3.18 (± 0.16)[a]	2.15 (± 0.13)[b]	1.92 (± 0.12)[b]	1.97 (± 0.12)[b]
	Schizochytrium	1.13 (± 0.02)[a]	0.42 (± 0.02)[b]	0.33 (± 0.07)[b]	0.30 (± 0.10)[b]
Zeaxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	3.4 (± 0.3)[a]	1.3 (± 0.06)[b]	0.8 (± 0.3)[bc]	0.4 (± 0.2)[c]
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-
Antheraxanthin	*Isochrysis*	-	-	-	-
	Nannochloropsis	-	-	-	-
	Phaeodactylum	-	-	-	-
	Schizochytrium	-	-	-	-

C	Microalgal species	Week 0	Week 4	Week 8	Week 12
Fucoxanthin	*Isochrysis*	12.93 (± 0.18)[a]	2.17 (± 0.03)[b]	0.43 (± 0.09)[c]	-
	Nannochloropsis	-	-	-	-
	Phaeodactylum	15.3 (± 1.2)[a]	6.3 (± 0.5)[b]	2.4 (± 0.2)[c]	0.88 (± 0.07)[c]
	Schizochytrium	-	-	-	-
Diatoxanthin	*Isochrysis*	3.32 (± 0.04)[a]	1.92 (± 0.02)[b]	1.46 (± 0.03)[c]	1.04 (± 0.10)[d]
	Nannochloropsis	-	-	-	-
	Phaeodactylum	3.6 (± 0.3)[a]	2.7 (± 0.2)[b]	2.33 (± 0.18)[b]	2.37 (± 0.19)[b]
	Schizochytrium	-	-	-	-

6.4. CONCLUSIONS

Tomato puree was enriched with biomass of three photoautotrophic microalgae (*Isochrysis, Nannochloropsis* and *Phaeodactylum*) and one heterotrophic microalga (*Schizochytrium*). The amount of n-3 LC-PUFA was in none of the tomato purees supplemented with different microalgae reduced by mechanical or thermal processing.

Independently of the applied processing intensity, the tomato purees supplemented with the heterotrophic microalga were less oxidatively stable compared to tomato purees supplemented with photoautotrophic microalgae. At 4 weeks storage at 37°C the primary oxidation products of tomato purees supplemented with *Schizochytrium* showed a maximum and by that time the secondary oxidation products were already highly increased, while the tomato purees supplemented with photoautotrophic microalgae showed at most an initiation of oxidation during 12 weeks of storage at 37°C. It was observed that a tomato puree, although rich in antioxidative carotenoids (e.g. lycopene), phenolic compounds, tocopherol and ascorbic acid, was not able to keep n-3 LC-PUFA derived from *Schizochytrium* biomass oxidatively stable.

The higher oxidative stability of tomato puree supplemented with photoautotrophic microalgae could mainly be attributed to the presence of carotenoids (xanthophylls, irrespective of type, and/or β-carotene) in the photoautotrophic microalgal biomass. Drastic degradations of these carotenoids in an early stage of storage confirmed this hypothesis. Phenolic compounds, tocopherols and ascorbic acid seemed to have a minor role in the maintenance of the oxidative stability.

Chapter 7:

IMPACT OF TYPE OF VEGETABLE PUREE ON THE OXIDATIVE STABILITY OF PUREES ENRICHED WITH N-3 LC-PUFA RICH MICROALGAE

7.1. INTRODUCTION

Chapter 6 has already shown a clear difference in the oxidative stability of tomato puree enriched with photoautotrophic and heterotrophic microalgal biomass. Tomato purees, rich in endogenous antioxidants, were not able to prevent the purees enriched with *Schizochytrium* from oxidation, while the tomato purees enriched with photoautotrophic microalgae showed at most an initiation of oxidation during 12 weeks of storage at 37°C. Possibly other vegetable purees, differing in type and amount of endogenous antioxidants, may show a different oxidative stability when enriched with n-3 LC-PUFA rich microalgal biomass.

Carotenoids, phenolic compounds and ascorbic acid are the main endogenous antioxidants in vegetables [223]. A combined effect of all these compounds determines the total antioxidant capacity [224]. A few studies determined the total antioxidant capacity of vegetables by measuring, amongst others, oxygen radical absorbance capacity (ORAC), FRAP and TEAC of different vegetables [225–230]. It should be mentioned that these methods only give an indication of the antioxidant capacity and that the value is highly influenced by the used method, vegetable cultivar and growth conditions. Based on these studies it could be concluded that more commonly used vegetables greatly varied in antioxidant capacity [225–230].

Ranawana *et al.* [231] fortified bread, rich in unsaturated corn oil, with freeze dried vegetables to improve its oxidative stability. All vegetables significantly increased the antioxidant potential of bread, with the highest increase in bread enriched with beetroot, followed by broccoli, followed by tomato and finally by carrot. Although all vegetables significantly increased the antioxidant potential, only beetroot and broccoli significantly increased the induction time of oxidation. Duthie *et al.* [232] enriched cooked turkey meat patties with vegetable powders. Enriching meat patties with carrots and swede did not improve the induction time of lipid oxidation, while broccoli, celery and beetroot slightly (not significantly) and tomato, spinach, onion and red pepper significantly

increased the induction time. Raikos *et al.* [233] incorporated fresh and freeze dried vegetables in mayonnaise. Incorporation of beetroot improved the oxidative stability, while no (positive or negative) effect on the oxidative stability of mayonnaise was observed when carrots or onions were incorporated. Incorporation of broccoli had a pro-oxidative effect, which is in contrast to the results of the previously mentioned studies. The varying results observed in the different studies, could possibly be attributed to differences in storage conditions, parameters used to monitor lipid oxidation or lipid oxidation mechanism.

In the study of Raikos *et al.* [233], incorporation of freeze dried vegetables resulted in a mayonnaise that was more prone to lipid oxidation compared to mayonnaise enriched with fresh vegetables, suggesting that processing may influence the antioxidant capacity of vegetables. Nicoli *et al.* [234] stated that the magnitude of antioxidant losses during processing depends on the food product and the processing method. Jimenez-Monreal *et al.* [235] studied the impact of 5 different processing methods in 20 different vegetables. The highest loss in free radical scavenging capacity was observed in boiled pepper (up to 75%), while frying artichoke only reduced the free radical scavenging capacity with 5%.

The impact of type of vegetable on the oxidative stability of n-3 LC-PUFA enriched vegetable purees has never been described before. The objective of this chapter was therefore to obtain more insight into the oxidative stability of different vegetable purees enriched with n-3 LC-PUFA rich photoautotrophic microalgal biomass (*Nannochloropsis*) on the one hand and with n-3 LC-PUFA rich heterotrophic microalgal biomass (*Schizochytrium*) on the other hand. Only one photoautotrophic microalgae was used as no differences in oxidative stability between the several photoautotrophic microalgae were observed in chapter 6. *Nannochloropsis* sp. was selected because of its potential shown in previous chapters. *Schizochytrium* was selected as heterotrophic microalgae because large differences in oxidative stability between photoautotrophic and heterotrophic microalgae were observed in chapters 4 and 6. The vegetables used in this chapter (broccoli, carrot and

tomato purees) were selected based on their commercial importance [236] as well as on their differences in endogenous antioxidants [232]. Although broccoli and tomato are both rich in carotenoids, phenolic compounds and vitamin C, their carotenoid profile differs [237,238]. Broccoli contains mainly β-carotene and lutein [238], while tomato contains mainly lycopene and β-carotene. Carrot contains lower amounts of phenolic compounds and vitamin C, but is rich in α- and β-carotene [239]. The vegetable purees were enriched to reach a concentration of 80 mg n-3 LC-PUFA/100 g enriched puree and characterized for their amount of n-3 LC-PUFA, carotenoids, phenolic compounds, ascorbic acid and oxidative stability and carotenoid degradation during 12 weeks of storage at 37°C. Furthermore, the impact of processing (high pressure homogenization, pasteurization or sterilization) was also investigated**.**

7.2. MATERIALS AND METHODS

7.2.1. MICROALGAL SPECIES

One photoautotrophic and one heterotrophic n-3 LC-PUFA rich microalga was used in this chapter. *Nannochloropsis* sp. biomass (photoautotrophic) was obtained as freeze dried biomass from Proviron (Hemiksem, Belgium) and *Schizochytrium* sp. freeze dried biomass (heterotrophic) from Bunge (St Just Desvern, Spain). Both biomasses were stored at -80°C until further use.

7.2.2. PREPARATION OF VEGETABLE PUREES

Broccoli (cultivar *Monrello*) and carrots (cultivar *Neria*) were obtained from REO Veiling Roeselare (Roeselare, Belgium). The broccoli and carrot puree was prepared according to the method described in section 5.2.2. The results of tomato purees presented in this chapter are those obtained in chapter 6. Although for clarity and easier interpretation, they are presented in this chapter as well.

7.2.3. EXPERIMENTAL SET-UP

The general experimental set-up of this chapter is shown in Figure 7.1. For *Nannochloropsis* and *Schizochytrium*, a broccoli, carrot and tomato puree was prepared according to the steps shown in Figure 5.2. According to previous chapters, a *2x2* experimental set-up was selected.

7.2.4. PREPARATION AND PROCESSING OF ENRICHED VEGETABLE PUREES

Preparation and further processing (high pressure homogenization, pasteurization and sterilization) of the enriched vegetable purees was performed according to section 5.2.4. Table 7.1 represents the pH and antioxidant composition of the vegetable purees.

Table 7.1. *pH and antioxidant composition of broccoli, carrot and tomato puree, mean (± SD). Statistical differences in are indicated with a different letter, α=0.05.*

	Broccoli	***Carrot***	***Tomato***
pH	6.44 (±0.17)^{a}	6.09 (±0.21)^{a}	4.38 (±0.15)^{b}
Carotenoids (10^{-2} mg/g vegetable puree)			
α-carotene	-	4.37 (±0.12)	-
β-carotene	0.61 (±0.02)^{c}	6.87 (±0.02)^{a}	1.12 (±<0.01)^{b}
Lutein	0.44 (±0.02)	-	-
Lycopene	-	-	5.21 (±<0.01)
Chlorophylls (10^{-2} mg/g vegetable puree)			
Chlorophyll a	2.20 (±<0.01)	-	-
Phenolic compounds (10^{-2} mg/g vegetable puree)	20.5 (±1.4)^{a}	4.08 (±0.09)^{c}	14.38 (±0.02)^{b}
Ascorbic acid (10^{-2} mg/g vegetable puree)	39.45 (±0.04)^{a}	3.4 (±0.6)^{c}	20 (±2)^{b}

7.2.5. ANALYSES

7.2.5.1. LIPID EXTRACTION

The lipids were extracted by a chloroform/methanol (1/1) extraction from the freeze dried puree as described in section 3.2.2.1. This analysis was performed in duplicate on each sample.

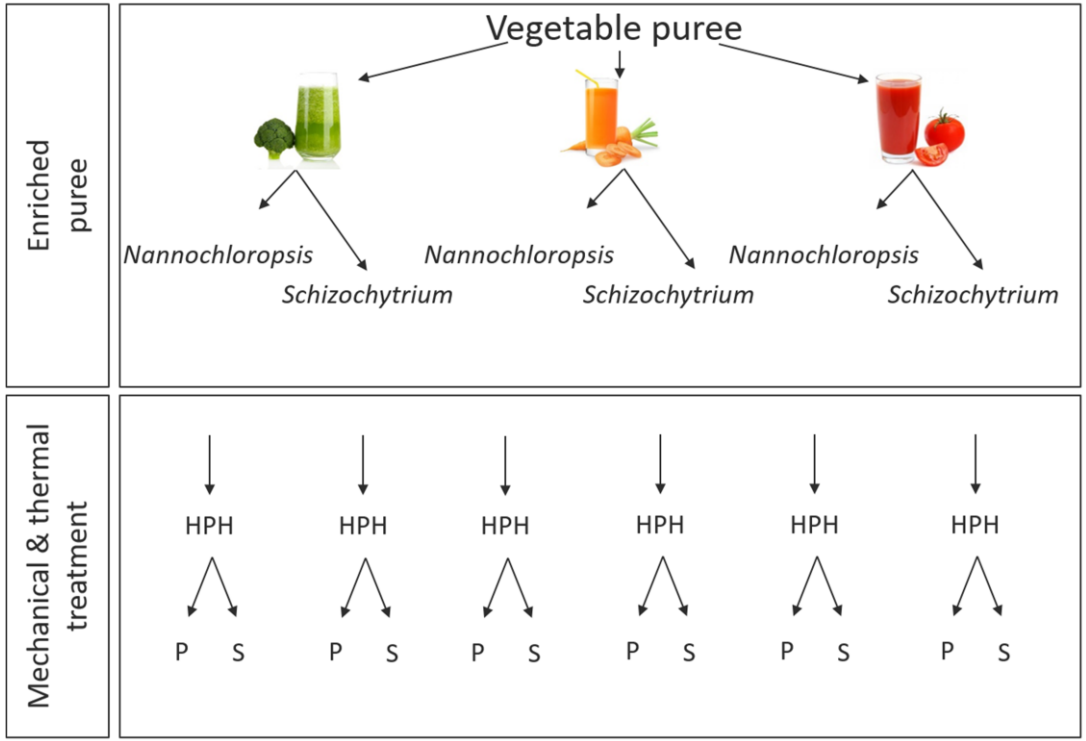


Figure 7.1. *Experimental set-up of this chapter in which HPH stands for high pressure homogenization, P for pasteurization and S for sterilization.. Each system was made in duplicate.*

7.2.5.2. DETERMINATION OF N-3 LC-PUFA CONTENT

The n-3 LC-PUFA content was determined by chromatographic separation and FID detection after methylation of the extracted lipids according to the method described in section 4.2.3.2. The analysis was performed in duplicate.

7.2.5.3. DETERMINATION OF PRIMARY OXIDATION PRODUCTS

The primary oxidation products were analyzed spectrophotometrically by the ferrous oxidation xylenol orange method according to the method of described in section 3.2.2.3. All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

7.2.5.4. DETERMINATION OF SECONDARY OXIDATION PRODUCTS

Volatile secondary oxidation products were analyzed with HS-SPME GC-MS according to section 3.2.2.7. The semi quantitative concentrations of the identified volatile compounds were calculated as the area of the volatile indicator compound divided by the response factor of the external standard hexanal (spiked in the corresponding vegetable puree). All data points were expressed as delta values, which is the relative increase or decrease compared to week zero, to compare the impact of oxidation during storage. All analyses were done in duplicate.

7.2.5.5. DETERMINATION OF CAROTENOIDS

The carotenoid content was determined by HPLC-PAD after extraction with acetone/methanol (7/3) as described in section 5.2.5.4. The analysis was performed in duplicate.

7.2.5.6. DETERMINATION OF TOTAL PHENOLIC COMPOUNDS

The determination of the phenolic content was done with the Folin-Ciocalteu reagent according to the method section 5.2.5.5. The analysis was performed in duplicate.

7.2.5.7. DETERMINATION OF ASCORBIC ACID

The ascorbic acid content was determined by HPLC coupled with fluorescence detection after a derivatization reaction according to Brown section 5.2.5.6. The analysis was performed in duplicate.

7.2.6. CORRECTION FOR PHYSICAL LOSSES

The values of all parameters were corrected for physical losses according to section 5.2.6. By this correction, physical losses occurring during processing, stickiness to glassware, mixer, etc., were left out of consideration.

7.2.7. STATISTICAL ANALYSIS

Results were statistically evaluated by a two way ANOVA (within each microalgal species) to consider the impact of vegetable puree and processing. The differences observed between the incorporation of *Nannochloropsis* and *Schizochytrium* will not be discussed in too much detail as this has been extensively described in chapter 6. The results of the storage experiment were evaluated by a two way ANOVA to consider the impact of storage time and type of vegetable puree for each processing step and microalgal species. The degradation of carotenoids (for each carotenoid individual) was evaluated by one way ANOVA to consider the impact of time for each enriched vegetable puree. The differences were determined by a *post hoc* Tukey test. All statistical tests were performed with $\alpha = 0.05$ (JMP Pro 14, SAS Institute Inc.).

7.3. RESULTS AND DISCUSSION

Vegetable purees enriched with *Nannochloropsis* on the one hand and *Schizochytrium* on the other will be discussed separately. The differences observed between the different microalgal species have already been extensively described in chapter 6.

7.3.1. VEGETABLE PUREES ENRICHED WITH PHOTOAUTOTROPHIC MICROALGAE

7.3.1.1. n-3 LC-PUFA CONTENT

Figure 7.2 shows the amount of n-3 LC-PUFA in the enriched vegetable purees after the different processing steps. Logically, the amount of n-3 LC-PUFA in the mixed purees was the added standardized amount of 80 mg n-3 LC-PUFA/100 g enriched purees. Processing did not statistically reduce the amount of n-3 LC-PUFA in none of the enriched vegetable purees. This observation is in line with the results described in chapters 5 and 6 on n-3 LC-PUFA rich photoautotrophic microalgae added to tomato puree and research of photoautotrophic microalgae in pasta[77] and dairy analogs[76].

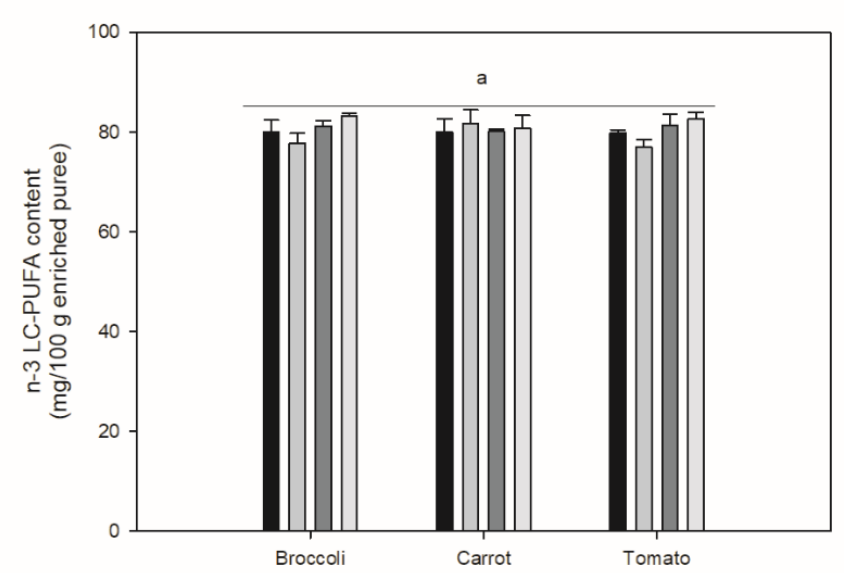


Figure 7.2. *Amount of n-3 LC-PUFA (expressed as mg/100 g enriched puree) after correction for physical losses in broccoli, carrot and tomato purees enriched with Nannochloropsis. The amount in the enriched puree was determined after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). No significant differences were observed, α=0.05.*

7.3.1.2. LIPID OXIDATION

Figure 7.3 shows the formation of primary and secondary lipid oxidation products during 12 weeks of storage at 37°C. In general, the formation of primary and secondary oxidation compounds was limited. Visually a small increase in oxidation products could be observed in the more intensively processed purees. However nor the primary oxidation products, nor the secondary oxidation products showed a significant increase during storage, except for the primary oxidation products of the sterilized enriched broccoli puree. The slight increase in peroxide value in the latter sample suggested only an indication of oxidation, as this trend was not yet observed in the secondary oxidation compounds.

Despite the large differences in the vegetable purees in terms of antioxidant capacity, no differences in oxidative stability were thus observed. This implies that the main reason for the oxidative stability is probably linked to the photoautotrophic microalga itself. The type of vegetable puree seemed rather subordinately when photoautotrophic microalgae were used.

The high oxidative stability of photoautotrophic microalgae in enriched tomato puree was already observed in chapters 5 and 6. In the study of Babuskin *et al.* [83], in chikkis, and in chapter 4, in aqueous model systems similar results in matrices with a lower antioxidant capacity could be observed. Mainly the endogenous antioxidants of the microalga itself thus appear to contribute to the high oxidative stability, so that variations in the antioxidant capacity of the vegetable puree are not observable.

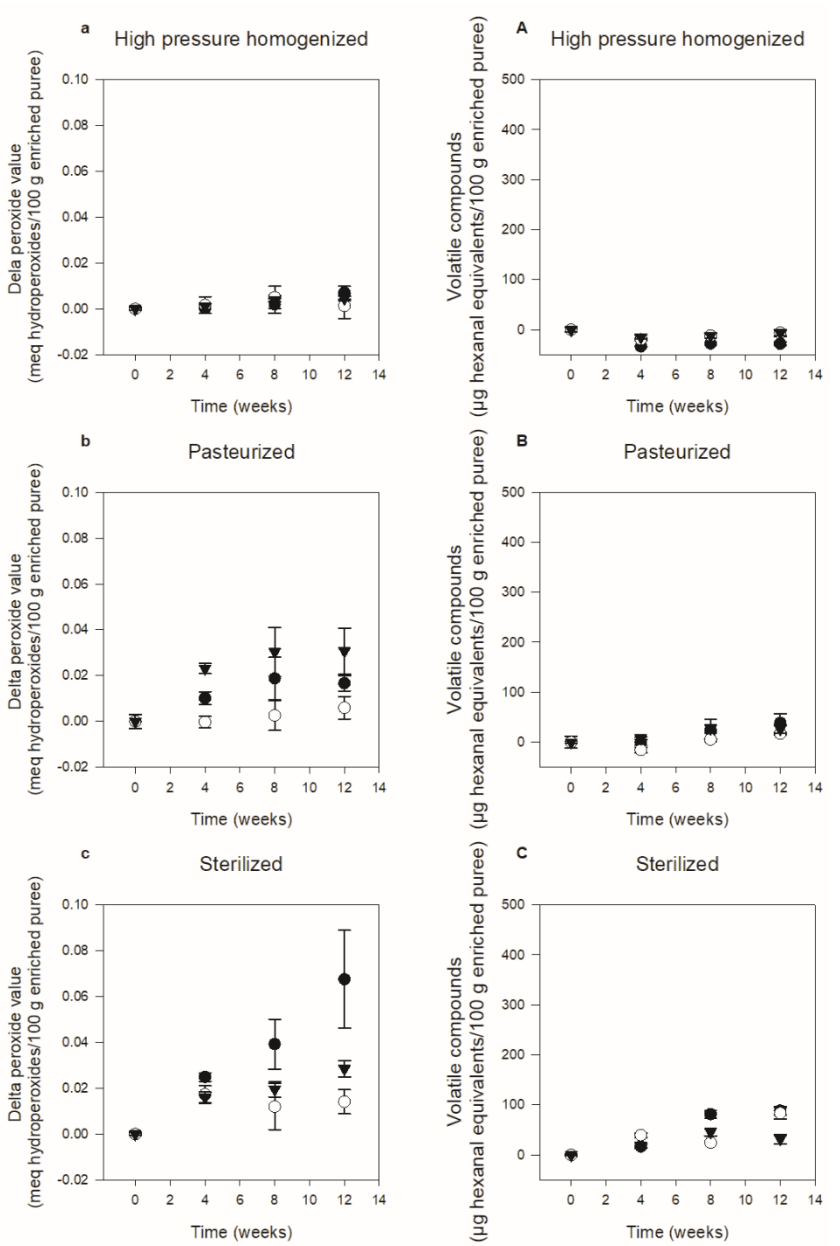


Figure 7.3. *Evolution of primary (small letter) and secondary (capital letter) oxidation products during 12 weeks of storage at 37°C for broccoli (●), carrot (○), tomato (▼) enriched with Nannochloropsis after high pressure homogenization (a & A), pasteurization (b & B) and sterilization (c & C), mean (± SD). The results are expressed as delta values, which is the relative increase or decrease compared to week zero.*

7.3.2. VEGETABLE PUREES ENRICHED WITH HETEROTROPHIC MICROALGAE

7.3.2.1. N-3 LC-PUFA CONTENT

Figure 7.4 represents the amount of n-3 LC-PUFA of vegetable purees enriched with *Schizochytrium*. Mixed purees logically contained the standardized amount of 80 mg n-3 LC-PUFA/100 g enriched puree. Mechanical and thermal processing did not reduce the amount of n-3 LC-PUFA in any of the vegetable purees.

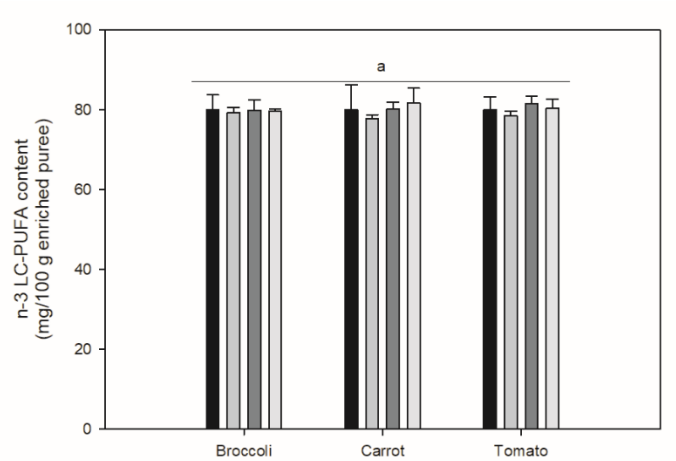


Figure 7.4. *Amount of n-3 LC-PUFA (expressed as mg/100 g enriched puree) after correction for physical losses in broccoli, carrot and tomato purees enriched with Schizochytrium. The amount in the enriched puree was determined after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). No significant differences were observed, α=0.05.*

This observation was not obvious as Lee *et al.* [94] reported reductions due to cooking up to 35% of the amount of n-3 LC-PUFA in meat products enriched with heterotrophic microalgal oil. This loss was however (at least partially) attributed to physical losses, for which we corrected for in this research.

7.3.2.2. LIPID OXIDATION

The formation of primary and secondary oxidation products during 12 weeks of storage at 37°C is shown in Figure 7.5. No significant increase in the peroxide value (primary oxidation products) in any of the enriched

purees, irrespective of processing, could be observed, except for pasteurized and sterilized enriched tomato puree at week 4 and pasteurized enriched broccoli puree at week 12. The absence of an increase in the peroxide value can imply that (i) the enriched puree is stable during storage or (ii) the puree is completely oxidized during storage and the peak maximum was located between two measuring points. Despite the fact that in most purees no significant change in peroxide value could be observed, a visual analysis of the graphs of enriched tomato purees showed an increased trend at week 4 followed by a decrease upon further storage, which indicates highly oxidized purees. A similar trend could be observed in high pressure homogenized enriched broccoli puree. Moreover, sterilized enriched broccoli puree contained large error bars, which is more often seen for oxidized purees.

The trends observed in the secondary oxidation compounds, being a significant increase at week 4 followed by a stagnation phase, were similar in all enriched vegetable purees. The increase in volatile compounds was significantly more pronounced in the enriched carrot puree. Again, no significant impact of processing was seen in the evolution of the secondary oxidation compounds as a function of time.

Based on the results of the secondary oxidation, it can thus be concluded that all enriched vegetable purees oxidized during storage. This implies that the peak maximum of the peroxide value was located between two measuring points, which led to the absence of significant differences in the primary oxidation. Despite the generally recognized antioxidant capacity of vegetable purees, none of them was thus able to maintain the n-3 LC-PUFA derived from *Schizochytrium* oxidatively stable. *Schizochytrium* incorporated in carrot puree was less stable compared to broccoli and tomato puree given the significantly lower amount of volatile compounds in the latter two.

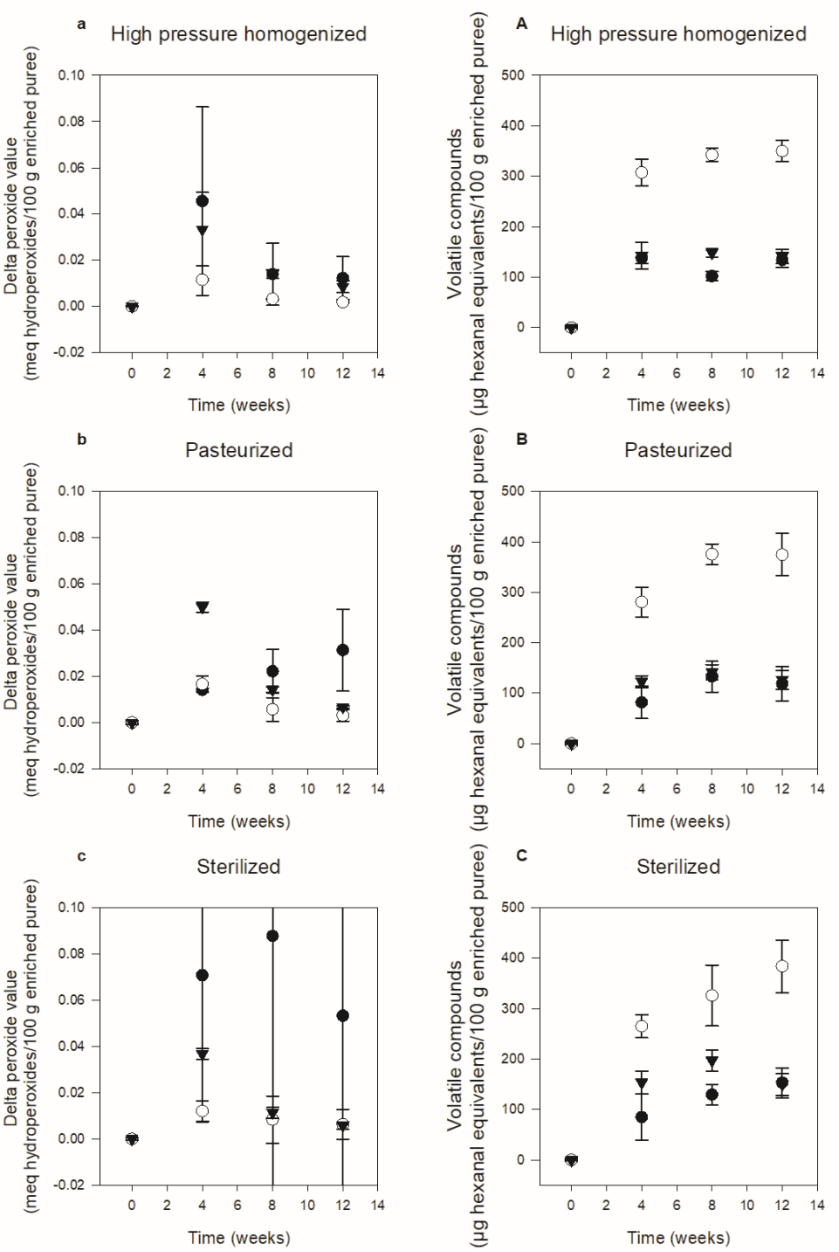


Figure 7.5. *Evolution of primary (small letter) and secondary (capital letter) oxidation products during 12 weeks of storage at 37°C for broccoli (●), carrot (○), tomato (▼) enriched with Schizochytrium after high pressure homogenization (a & A), pasteurization (b & B) and sterilization (c & C) , mean (± SD). The results are expressed as delta values, which is the relative increase or decrease compared to week zero.*

These results are in agreement with the results of Ranawana *et al.*[231] and Duthie *et al.* [232], who already showed the higher antioxidant capacity of broccoli and tomato compared to carrot in respectively bread and meat patties. Probably, the lower antioxidant capacity of the carrot puree is the main explanation for the observed differences. To confirm this hypothesis, the main endogenous antioxidants originating from the vegetable purees are discussed in section 7.3.2.3. Their link with the results of the oxidative stability is discussed in section 7.3.2.4.

7.3.2.3. INFLUENCING FACTORS

i. *CAROTENOIDS*

Figure 7.6a-d represents the carotenoids present in the enriched vegetable purees. Since *Schizochytrium* did not contain carotenoids, the enriched purees only contained carotenoids originating from the vegetable puree itself (Table 7.1 and 7.2). Enriched broccoli puree contained lutein (0.5 mg/100 g enriched puree) and β-carotene (0.75 mg/100 g enriched puree), enriched carrot puree contained α-carotene (6.3 mg/100 g enriched puree) and β-carotene (9.5 mg/100 g enriched puree) and enriched tomato puree contained lycopene (5 mg/100 g enriched puree) and β-carotene (0.9 mg/100 g enriched puree). None of the carotenoids was significantly influenced by processing.

ii. *TOTAL PHENOLIC COMPOUNDS*

The phenolic compounds present in the enriched purees are shown in Figure 7.6e. A significant impact of type of vegetable puree could be observed. Enriched broccoli puree contained the highest amount of phenolic compounds (17 mg GAE/100 g enriched puree) followed by enriched tomato puree (14 mg GAE/100 g enriched puree) and followed by enriched carrot puree (4 mg GAE/100 g enriched puree). The lower concentration in enriched tomato and carrot puree can be attributed to the lower concentrations in the vegetable puree itself, since all purees were enriched with the same amount of *Schizochytrium* and the amount of total phenolic compounds in *Schizochytrium* was negligible.

Table 7.2. Theoretical calculated values of n-3 LC-PUFA, free fatty acids and antioxidants (mg/100 g enriched puree) of broccoli, carrot and tomato purees enriched with Schizochytrium, mean (±SD).

	Broccoli	*Carrot*	*Tomato*
n-3 LC-PUFA (mg/100 g enriched puree)	81.0 (± 3)	81.0 (± 3)	81.0 (± 3)
Free fatty acid (mg/100 g enriched puree)	1.34 (± 0.10)	1.34 (± 0.10)	1.34 (± 0.10)
Carotenoids (mg/100 g enriched puree)			
β-carotene	0.58 (± 0.02)	6.49 (± 0.02)	1.00 (±< 0.01)
Lutein	0.42 (± 0.02)		
α-carotene	-	4.13 (± 0.11)	-
Lycopene	-	-	4.65 (± 0.02)
Phenolic compounds (mg/100 g enriched puree)	20.0 (± 1.3)	4.49 (± 0.10)	13.48 (± 0.05)
Tocopherols (mg/100 g enriched puree)	0.41 (± 0.10)	0.22 (± 0.06)	0.58 (± 0.14)
Ascorbic Acid (mg/100 g enriched puree)	37 (± 4)	3.3 (± 0.7)	19 (± 2)

The results were in line with the results of Zhou & Yu [229] who also showed that freeze dried broccoli has a tree times higher total phenolic content compared to freeze dried tomato and a six times higher content compared to freeze dried carrots. In none of the purees, a significant impact of processing was observed which was in accordance with chapter 6 and the study of Dewanto *et al.*[206] where comparable processing steps were executed.

iii. *ASCORBIC ACID*

Figure 7.6f shows the amount of ascorbic acid in the enriched vegetable purees. A significant impact of processing and a significant impact of type of vegetable was observed. Thermal processing reduced the amount of ascorbic acid in the enriched purees drastically. This was observed in all enriched vegetable purees. In enriched carrot puree, no significant difference was observed between pasteurization and sterilization, while in enriched broccoli and tomato puree sterilization led to a supplementary reduction compared to pasteurization. In literature, ascorbic acid was already described as a thermolabile compound [219].

The differences observed in the initial amount of ascorbic acid can be attributed to the different amounts in the vegetable purees as the ascorbic acid only originated from the vegetable puree itself. Enriched broccoli puree contained a significantly higher amount (26 mg ascorbic acid/100 g mixed enriched puree) compared to enriched tomato puree (19 mg ascorbic acid/100 g mixed enriched puree). The lowest amount was observed in enriched carrot puree (4 mg ascorbic acid/100 g mixed enriched puree). These results are in line with the results obtained by Szeto *et al.* [226].

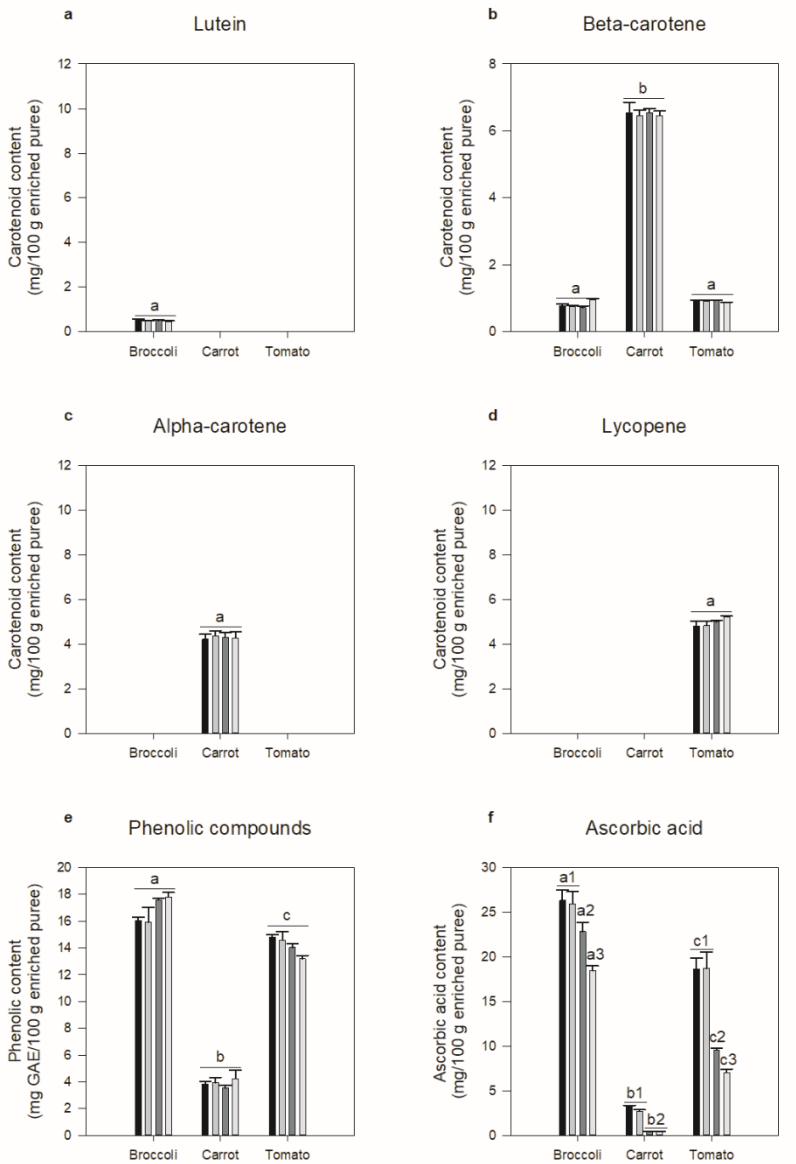


Figure 7.6. *Antioxidant composition, carotenoids (a-d), lutein (a), β-carotene (b), α-carotene (c), lycopene (d), total phenolic compounds (e) and ascorbic acid (f) for vegetable purees enriched with Schizochytrium after mixing (●), high pressure homogenization (●), pasteurization (●), and sterilization (●), mean (± SD). For carotenoids and phenolic compounds, statistical differences are indicated with a different letter, α=0.05. For ascorbic acid, differences within a treatment are indicated with a different letter, while differences within a type of vegetable are indicated with a different number, α=0.05.*

7.3.2.4. RELATION BETWEEN OXIDATIVE STABILITY AND INFLUENCING FACTORS

Incorporation of heterotrophic microalgae implies the absence of antioxidants originating from the microalga itself. Therefore, the antioxidants derived from the food product play an important role in the determination of the oxidative stability of enriched products. Large differences in oxidative stability were observed between the enriched broccoli and tomato puree on the one hand and enriched carrot puree on the other hand (7.3.2.2). In parallel, differences in antioxidants, known for their impact on the oxidative stability, were seen (7.3.2.3).

At first sight, the results of the carotenoids present in the enriched purees seemed contradictory to the observed oxidative stability during storage. However, not only the total amount of carotenoids is crucial in the determination of the oxidative stability. It can be hypothesized that the lower total carotenoid concentration in enriched broccoli and tomato purees was (partially) compensated by the type of carotenoid [240]. In literature, different studies showed varying results, although all of them relied on fast methods of antioxidative capacity and none of them really monitored lipid oxidation like in this chapter. Woodall *et al.* [241] showed a higher antioxidant capacity of lycopene compared to β-carotene based on the Fenton reaction. Müller *et al.* [242] screened the capacity of carotenoids, among which lutein, β-carotene, α-carotene and lycopene, with different assays (TEAC, FRAP and luminol-chemiluminescence based peroxyl radical scavenging assay (LPSC)). The used assays, based on a variety of principles, gave different results. Measured by TEAC, lycopene showed a higher potential in scavenging synthetic radicals compared to α-carotene and β-carotene. Lutein showed the lowest potential. Based on the FRAP method, lycopene and lutein showed a higher ferric reducing potential. The LPSC assay in its turn showed a higher peroxyl radical scavenging capacity for lutein, followed by α-carotene and β-carotene and followed by lycopene. As the different methods often gave different results, a weighed value, based on the different used assays, was calculated. Based on the latter, a higher antioxidant potential was obtained for lycopene and lutein compared to α-carotene and β-carotene. This supports that not

only the total amount of carotenoids should be taken into consideration to make the link with the results of oxidative stability. Moreover, synergistic effects between different carotenoids might have occurred [124].

Additionally, the amount of phenolic compounds and ascorbic acid was significantly lower in the enriched carrot puree, which showed the lowest oxidative stability during storage. This indicates a possible role of these antioxidants in the prevention of lipid oxidation. However, a crucial role of ascorbic acid seems doubtable as almost no significant impact of processing on the oxidative stability was observed while the amount of ascorbic acid was highly influenced by processing.

Moreover, pro-oxidative effects of α- and β-carotene at higher concentrations can also possibly explain the lower oxidative stability of enriched carrot puree [243,244]. The conditions at which these effects occur are hard to determine as the effect is influenced by environmental factors and the composition of the sample [134,139].

Based on the previous results, three hypotheses could be formulated to explain the higher oxidative stability of broccoli and tomato compared to carrot: (i) the higher antioxidant capacity of lycopene and lutein compared to α- and β-carotene, (ii) the higher concentration of phenolic compounds or (iii) or the high concentrations of α- and β-carotene resulting in pro-oxidative effects. A synergistic effect of different antioxidants [124] may be a fourth explanation.

As in chapters 5 and 6, the evolution of the carotenoids during storage was investigated. Tables 7.3a–c show the evolution of carotenoids originating from the vegetable purees enriched with *Schizochytrium.*

In general, all carotenoids showed a decrease over time in all enriched purees. This degradation can be explained by the contribution of carotenoids in the reaction with radicals or oxygen. Irrespective of the processing intensity, broccoli purees enriched with *Schizochytrium* showed a reduction up to 70% of β-carotene during 12 weeks of storage, while α- and β-carotene of carrot purees enriched with *Schizochytrium* were reduced up to 65 and 60%, respectively. Lycopene and β-carotene

derived from tomato purees enriched with *Schizochytrium* showed a degradation up to 65 and 75% respectively. A significant reduction was mostly observed from week 4 of storage.

The degradation of α- and β-carotene in carrot puree enriched with *Schizochytrium* was less pronounced compared to the reduction of β-carotene in enriched broccoli puree and lycopene and β-carotene in enriched tomato puree, which could possibly be linked with the lower oxidative stability of the former. In broccoli β-carotene is mainly located in the chromoplasts [245], while in tomato and carrot it is often located in large solid-crystalline aggregates, which however differ in size and origin [245] between both vegetables. A reduction in availability and thus antioxidant capacity [245] depending on the deposition form may explain the difference β-carotene degradation. The hypothesis of the impact of carotenoid deposition on the oxidative stability should be further investigated. In the current research, the impact of carotenoid deposition in vegetable foods has mainly been investigated in terms of bioavailability and bioaccessibility. Moreover, processing of the vegetables can influence the internal structures and thus change the availability [246–248].

The carotenoids degraded in a later stage of storage if the vegetable puree was enriched with *Nannochloropsis*. Additionally, the degradation was less pronounced compared to purees enriched with *Schizochytrium.* This could be explained by the presence of zeaxanthin and antheraxanthin derived from *Nannochloropsis.* These carotenoids degraded first and more drastic (up to 85 and 100% respectively, results not shown). These results suggest that if no carotenoids derived from the microalgae are present in the enriched puree, the degradation of carotenoids from the vegetable puree is larger. All these results are in accordance with the results of chapters 5 and 6.

As all carotenoids from the vegetable puree degrade, their antioxidative effect is proven, but the results were insufficient to draw a conclusion on which of the proposed hypotheses is most likely.

Table 7.3. *Evolution of the carotenoids of broccoli, carrot and tomato purees supplemented with Schizochytrium after high pressure homogenization (a), pasteurization (b) and sterilization (c) during 12 weeks of storage, mean (± SD). Statistical differences in function of time (per enriched puree) are indicated with a different letter, α=0.05.*

a	Vegetable puree	High pressure homogenized			
		Week 0	Week 4	Week 8	Week 12
β-carotene	*Broccoli*	0.475 (± 0.009)^{a}	0.33 (± 0.04)^{ab}	0.27 (± 0.07)^{b}	0.20 (± 0.04)^{b}
	Carrot	6.46 (± 0.16)^{a}	4.3 (± 0.3)^{b}	3.8 (± 0.3)^{b}	3.4 (± 0.8)^{b}
	Tomato	1.13 (± 0.04)^{a}	0.5 (± 0.2)^{b}	0.31 (± 0.06)^{b}	0.27 (± 0.03)^{b}
α-carotene	*Broccoli*	-	-	-	-
	Carrot	4.4 (± 0.2)^{a}	2.5 (± 0.2)^{b}	2.3 (± 0.2)^{b}	2.2 (± 0.5)^{b}
	Tomato	-	-	-	-
Lycopene	*Broccoli*	-	-	-	-
	Carrot	-	-	-	-
	Tomato	4.6 (± 0.3)^{a}	3.3 (± 0.6)^{ab}	2.6 (±0.4)^{b}	2.31 (± 0.16)^{b}
Lutein	Broccoli	0.30 (± 0.02)^{a}	0.08 (± 0.02)^{b}	0.05 (± 0.02)^{b}	-
	Carrot	-	-	-	-
	Tomato	-	-	-	-

b

	Vegetable puree	Pasteurized			
		Week 0	Week 4	Week 8	Week 12
β-carotene	*Broccoli*	0.44 (± 0.09)[a]	0.49 (± 0.13)[a]	0.32 (± 0.06)[a]	0.16 (± 0.04)[a]
	Carrot	6.54 (± 0.12)[a]	4.6 (± 0.6)[ab]	3.9 (± 0.8)[b]	3.3 (± 0.8)[b]
	Tomato	1.15 (± 0.04)[a]	0.43 (± 0.02)[b]	0.26 (± 0.02)[c]	0.21 (± 0.06)[c]
α-carotene	*Broccoli*	-	-	-	-
	Carrot	4.3 (± 0.2)[a]	2.5 (± 0.2)[b]	2.1 (± 0.5) [b]	2.0 (± 0.5)[b]
	Tomato	-	-	-	-
Lycopene	*Broccoli*	-	-	-	-
	Carrot	-	-	-	-
	Tomato	4.9 (± 0.2)[a]	2.94 (± 0.17)[b]	2.14 (± 0.06)[bc]	1.7 (± 0.5)[c]
Lutein	Broccoli	0.31 (± 0.02)[a]	0.18 (± 0.11)[ab]	0.063 (± 0.011)[b]	-
	Carrot	-	-	-	-
	Tomato	-	-	-	-

c	Vegetable puree	Sterilized			
		Week 0	Week 4	Week 8	Week 12
β-carotene	*Broccoli*	0.50 (± 0.02)^{a}	0.26 (± 0.12)^{ab}	0.21 (± 0.10)^{ab}	0.15 (± 0.07)^{b}
	Carrot	6.54 (± 0.14)^{a}	4.5 (± 0.6)^{b}	3.7 (± 0.6)^{bc}	2.6 (± 0.6)^{c}
	Tomato	1.13 (± 0.02)^{a}	0.42 (± 0.02)^{b}	0.33 (± 0.07)^{b}	0.30 (± 0.10)^{b}
α-carotene	*Broccoli*	-	-	-	-
	Carrot	4.2 (± 0.3)^{a}	2.5 (± 0.3)^{b}	2.1 (± 0.5)^{b}	1.5 (± 0.3)^{b}
	Tomato	-	-	-	-
Lycopene	*Broccoli*	-	-	-	-
	Carrot	-	-	-	-
	Tomato	4.95 (± 0.09)^{a}	2.85 (± 0.09)^{b}	2.5 (± 0.3)^{b}	2.4 (± 0.7)^{b}
Lutein	Broccoli	0.29 (± 0.02)^{a}	0.08 (± 0.02)^{b}	0.03 (± 0.03)^{b}	-
	Carrot	-	-	-	-
	Tomato	-	-	-	-

7.4. CONCLUSIONS

No significant differences in the amount of n-3 LC-PUFA in none of the enriched purees (broccoli, carrot or tomato puree) with the photoautotrophic microalga *Nannochloropsis* or heterotrophic microalga *Schizochytrium* was observed, independently of the applied processing intensity.

Despite the large differences in terms of antioxidant capacity of the vegetable purees, no differences in oxidative stability were observed if *Nannochloropsis* was incorporated. This implies that the main reason for the oxidative stability are the antioxidants of the photoautotrophic microalga themselves. The type of vegetable puree seemed rather subordinate when photoautotrophic microalgae were used.

On the other hand, all vegetable purees enriched with *Schizochytrium* were prone to oxidation. The incorporation of *Schizochytrium* implies the absence of antioxidants originating from the microalga itself. Therefore, the antioxidants derived from the food product played a more pronounced role in the determination of the oxidative stability of enriched products. A higher oxidative stability of enriched broccoli and tomato puree was observed compared to enriched carrot puree, which could be attributed to (i) the higher antioxidant capacity of lycopene and lutein compared to α- and β-carotene and/or a different carotenoid deposition form, (ii) the higher concentration of phenolic compounds, (iii) the high concentrations of α- and β-carotene resulting in pro-oxidative effects or (iv) synergistic effects between different antioxidants.

Chapter 8:

THE POTENTIAL OF N-3 LC-PUFA RICH MICROALGAE IN VEGETABLE PUREES:

GENERAL CONCLUSIONS & PERSPECTIVES FOR FUTURE WORK

The omega-3 long chain poly-unsaturated fatty acids (n-3 LC-PUFA), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3), are getting more and more attention since they have proven their beneficial health effects in brain development and the prevention of cardiovascular diseases [1]. The Food and Agricultural Organization of the United Nations [2] recommends an intake of 250 mg n-3 LC-PUFA/day. Fish is the most well-known source of n-3 LC-PUFA, although it is under pressure due to a reducing stock and the accumulation of toxic compounds (PCB's, dioxins, lead,…) [3]. Microalgae, the primary producers of n-3 LC-PUFA, have already shown their potential as an alternative, and even vegetarian and vegan, source of n-3 LC-PUFA [4].

Limited research has been performed on food products enriched with n-3 LC-PUFA rich microalgae. This research has only targeted a small range of food products, mainly carbohydrate-rich (bread, pasta, biscuits) and protein-rich (dairy, meat, fish and egg products) food products (chapter 1) and did not study the oxidative stability of these food products. The latter seems to be in contradiction with the fact that n-3 LC-PUFA are highly susceptible to lipid oxidation, which should be avoided, since it results in an undesired flavor, the presence of toxic compounds and loss of nutritional value [5].

The aim of this PhD was to investigate in-depth the potential of microalgae as an alternative source of n-3 LC-PUFA in vegetable-based products. These products are essential in a healthy diet as they contain high value compounds like fibers and antioxidants [6]. These antioxidants could furthermore stabilize the n-3 LC-PUFA against oxidation. The focus in this PhD was especially, on the impact of dosage form, microalgal species and type of vegetable puree on the n-3 LC-PUFA and endogenous antioxidant concentration and on the oxidative stability of enriched vegetable purees. In the next paragraphs, the conclusions about these aspects are summarized. At the end of this chapter, some future research possibilities are suggested.

8.1. GENERAL CONCLUSION

8.1.1. N-3 LC-PUFA ENRICHMENT

Throughout this PhD, a fixed concentration of 80 mg n-3 LC-PUFA was added to 100 g model suspension (chapter 4) or vegetable puree (chapters 5 – 7) by adapting the incorporation concentration of the n-3 LC-PUFA rich microalgae. At this fixed concentration, the product can be labelled as 'a product rich in omega-3' [74]. Physical losses, lipid oxidation and thermal degradation can reduce the amount of n-3 LC-PUFA during production [182] of enriched purees. These aspects are discussed in more detail in each of the following sections.

8.1.1.1. PHYSICAL LOSSES

Physical losses during processing were typically observed by stickiness to glassware, mixer, tubes of high pressure homogenizer, etc. These losses were primarily dependent on the dosage form (chapter 5). No impact of microalgal species (chapters 4 and 6) or type of vegetable puree (chapter 7) was observed.

In chapter 5, physical losses of n-3 LC-PUFA up to 50% were observed in tomato purees enriched with *Nannochloropsis* oil. This was five times higher compared to the purees supplemented with *Nannochloropsis* biomass due to the high viscosity of the former. From a practical and sustainability point of view, a lower usability of oil as dosage form was thus concluded. Physical losses result in the need for higher incorporation concentrations to obtain the desired final concentration of 80 mg n-3 LC-PUFA/100 g enriched puree, which leads to a higher cost. Due to the use of biomass, only small physical losses were observed in chapters 4, 6 and 7.

To be able to investigate a possible decrease due to breakdown of the compounds during processing, all results were throughout this PhD corrected for physical losses by using a correction factor. After this correction, no significant differences were observed in the amount of n-3 LC-PUFA in the enriched purees of chapters 5, 6 and 7. Processing

intensities typical for vegetable-based products did thus not influence the amount of n-3 LC-PUFA in the enriched purees. On the contrary, a reduced amount of n-3 LC-PUFA in enriched suspensions was observed in chapter 4. This reduction was less than 20% for the suspensions enriched with *Isochrysis, Nannochloropsis, Phaeodactylum* or *Schizochytrium*, but was more pronounced for systems enriched with *Porphyridium* with a high free fatty acid content. This reduction could have been caused by lipid oxidation or thermal degradation.

8.1.1.2. LIPID OXIDATION

A reduction in n-3 LC-PUFA content by extended lipid oxidation is based on the loss of initial substrate. It is however known that lipid oxidation does not correlate well with fatty acid loss, due to the limited sensitivity of the analytical method for fatty acid determination [145]. This was confirmed in chapters 6 and 7 where extended lipid oxidation in purees enriched with *Schizochytrium* was observed, without significant reductions in the amount of n-3 LC-PUFA. The lipid oxidation observed in chapter 4 was also not sufficient to explain the observed reduction in n-3 LC-PUFA.

8.1.1.3. THERMAL DEGRADATION

The reductions in n-3 LC-PUFA content observed in chapter 4 could be attributed to thermal degradation, possibly combined with extended oxidation in the case of heterotrophic microalgae. Thermal alterations, caused by non-oxidative as well as oxidative reactions, were (indicatively) detected in chapter 4. A pronounced increase in polymer content was observed in the model suspension enriched with *Porphyridium*, which showed the largest reduction in n-3 LC-PUFA. Additionally, this suspension contained the largest free fatty acid concentration. The latter could be related to the degree of lipid polymer formation, since it was shown that the use of *Porphyridium* with a lower initial free fatty acid content resulted in a lower reduction of n-3 LC-PUFA. In contrast, tomato puree enriched with a high free fatty acid containing *Isochrysis* (chapter

6) showed no significant reduction in n-3 LC-PUFA content. This indicated a protective role of the tomato puree against thermal degradation.

8.1.2. ENDOGENOUS ANTIOXIDANTS

Different endogenous antioxidants, originating from the microalgae as well as from the vegetable puree, were present in the enriched purees.

In none of the vegetable purees from chapters 5 – 7, the different processing steps influenced the amounts of most of the carotenoids (lycopene, β-carotene, α-carotene, zeaxanthin, lutein, diatoxanthin and echinenone). Only the amounts of antheraxanthin, violaxanthin and fucoxanthin decreased limitedly but significantly by thermal processing, and this in accordance to literature [204]. The presence of one or more epoxy groups is the predominant factor in reducing the thermal stability of the second group of carotenoids.

No significant impact of processing on the amount of phenolic compounds and tocopherols was observed throughout this PhD. In literature contradictory results have been found: either increasing or decreasing amounts, or no significant effects of processing were observed depending on the study [206,218,219].

Thermal processing drastically reduced the amount of ascorbic acid in the enriched purees. In most enriched purees sterilization led to a supplementary reduction compared to pasteurization. This coincides with literature in which ascorbic acid has already been described as a thermolabile compound [219,221].

8.1.3. OXIDATIVE STABILITY

Incorporation of n-3 LC-PUFA in products implies an increased susceptibility to lipid oxidation. During this PhD, the oxidative stability of the enriched products was investigated during 12 weeks of storage at 37°C. Primary oxidation was measured with the method optimized in chapter 3. The conclusions reached throughout chapters 5 – 7 were independent of the applied processing intensity.

Photoautotrophic microalgae were shown to be the most appropriate species in order to create an n-3 LC-PUFA enriched vegetable puree with a high oxidative stability (chapters 5 – 7). No significant increase was observed in oxidation parameters of vegetable purees enriched with photoautotrophic biomass, irrespective of the photoautotrophic microalgal species, while vegetable purees enriched with heterotrophic microalgae or fish oil were already oxidized at week 4. Drastic degradations (up to 100%) of the carotenoids derived from the photoautotrophic microalgae in an early stage of storage were observed. The carotenoids derived from the vegetable puree on the other hand, only showed a significant decrease in the later stages of the storage period. Moreover, the degradation was much lower (up to 45%) compared to the reductions observed in the carotenoids derived from the photoautotrophic microalgae. Based on all the above, it was hypothesized that the endogenous carotenoids derived from the photoautotrophic microalgae ensured their oxidative stability, although the exact mechanism is still unclear. The strong antioxidant capacity of specific carotenoids of the photoautotrophic microalgae (xanthophylls, irrespective of type, and/or β-carotene) and/or their close location to the n-3 LC-PUFA (when using photoautotrophic microalgae, carotenoids and n-3 LC-PUFA are located in the same cell or even cell organelle) may lead to the high oxidative stability. The role of tocopherols, ascorbic acid, phenolic compounds and free fatty acids on the oxidative stability appears to be minor, as often higher (lower in the case of free fatty acids) amounts were found in purees with a lower oxidative stability.

For photoautotrophic microalgae, intact or (partially) disrupted biomass were the preferred dosage forms. They performed slightly better compared to purees enriched with *Nannochloropsis* oil. This could primarily be attributed to the higher carotenoid content in the biomass compared to the oil (chapter 5). No differences in oxidative stability were observed if vegetable purees with a different antioxidant profile were enriched with photoautotrophic microalgae. Broccoli, carrot and tomato puree enriched with *Nannochloropsis* showed no significant differences in oxidative stability, which implies that the antioxidants of the

photoautotrophic microalgae were the main reason for their high oxidative stability (chapter 7).

It was observed that a vegetable puree, although rich in antioxidative carotenoids (e.g. lycopene), phenolic compounds and ascorbic acid, was not able to keep n-3 LC-PUFA derived from heterotrophic *Schizochytrium* biomass or fish oil oxidatively stable (chapters 5 – 7). In these systems, the antioxidants derived from the food product played a more pronounced role in the determination of the oxidative stability. Larger reductions (up to 75%) of the carotenoids of the vegetable puree were observed compared to purees enriched with photoautotrophic microalgae (chapter 7). The carotenoids of the vegetable purees degraded during storage, which indicates that they function as antioxidant and thus could reach the location where lipid oxidation occurred. A difference in oxidative stability between the different vegetable purees was also observed. The higher oxidative stability of broccoli and tomato puree enriched with *Schizochytrium* compared to the enriched carrot puree could be attributed to (i) the higher antioxidant capacity of lycopene and lutein compared to α- and β-carotene, (ii) the higher concentration of phenolic compounds, (iii) the high concentrations of α- and β-carotene resulting in pro-oxidative effects or (iv) synergistic effects between different antioxidants. Comparison of incorporation of *Schizochytrium* biomass in model suspensions (chapter 4) and vegetable puree (chapter 7) can exclude hypothesis (iii), as carrot puree showed a higher oxidative stability compared to aqueous model systems.

8.1.3.1. STATISTICAL MODEL

In order to support the hypothesis that primarily the carotenoids derived from the photoautotrophic microalgae improved the oxidative stability, a forward multiple linear model was built, using the data obtained in chapters 5 – 7. The data of chapter 4 were not included in the model as only carotenoids and no other antioxidants were measured.

The model presents the relationship between the degree of lipid oxidation (dependent variable), and different independent variables (free

fatty acids, total phenolic compounds, tocopherols, chlorophyll a, chlorophyll c2, ascorbic acid, lycopene, α-carotene, β-carotene, lutein, zeaxanthin, fucoxanthin, antheraxanthin, diatoxanthin, violaxanthin and echinenone). The amount of volatile compounds at week 12 was chosen as the best representation of the degree of lipid oxidation. This value was measured at the end of the storage period and is a better marker compared to the peroxide value. The latter can already have low values at this time, while the sample is completely oxidized. The regression coefficient of each of the independent variables represents its influence on the dependent variable.

The model was built by a stepwise regression of forward selection. In each step, an independent variable was considered for addition to the model. Independent variables were added in descending order of F-value. The final model was obtained if all variables with a p-value lower than 0.05 were entered. The multiple linear regression was performed with JMP Pro 12.1 (SAS Institute Inc., Cary, USA).

In the final model (equation 8.1), five variables were retained: zeaxanthin (p-value < 0.001), fucoxanthin (p-value < 0.001), antheraxanthin (p-value < 0.001), α-carotene (p-value < 0.001) and lycopene (p-value = 0.023). These significant variables explained 82.3% (adjusted R^2) of the variance observed in the oxidative stability. All other tested variables had a p-value larger than 0.18, which indicated that none of them had a significant effect on the oxidative stability.

$$Y_{\text{Degree of lipid oxidation}} =$$

$$168.64 - 38.78\, X_{\text{zeaxanthin}} - 5.50\, X_{\text{fucoxanthin}} - 43.37\, X_{\text{antheraxanthin}} - 6.62\, X_{\text{lycopene}} + 28.26\, X_{\alpha\text{-carotene}} \qquad (8.1)$$

All significant variables were carotenoids, which confirms the importance of these compounds to improve the oxidative stability of enriched purees. Zeaxanthin, fucoxanthin and antheraxanthin are carotenoids derived from the photoautotrophic microalgae. They have a negative coefficient in the estimated model, which shows their capacity to improve the oxidative stability. Based on their coefficient, zeaxanthin (-38.78) and

antheraxanthin (-43.37) were of larger importance to improve the oxidative stability compared to fucoxanthin (-5.5). Although, no differences in oxidative stability between purees enriched with *Nannochloropsis* (zeaxanthin and antheraxanthin) on the one hand and *Isochrysis and Phaeodactylum* (fucoxanthin) on the other were observed. This can be explained by the higher concentrations of fucoxanthin in the latter. Additionally, lycopene (-6.62), derived from tomato puree, showed an antioxidant capacity. Contrary, α-carotene derived from carrot puree showed pro-oxidant properties (28.26), although, it can be doubted whether or not a pro-oxidant effect of α-carotene is the primary explanation. Based on previous findings (comparison of chapter 4 and 7), it seems that the absence of strong antioxidants in carrot puree rather than pro-oxidant effects of α-carotene is the explanation for the positive coefficient.

The statistical model confirmed the hypothesized important role of carotenoids in the maintenance of the oxidative stability. A role of free fatty acids, phenolic compounds, tocopherols, ascorbic acid and chlorophylls could not be excluded, although it seems rather a subordinate role.

8.1.3.2. EXPLANATION OF THE HIGHER ANTIOXIDANT CAPACITY OF CAROTENOIDS

One of the major theories predicting the antioxidant capacity of a compound is the polar paradox. This paradox states that non-polar antioxidants are more effective in a polar environment compared to their polar variants, whereas polar antioxidants are more active in bulk lipids [249]. Our enriched vegetable purees consist of an aqueous environment (polar) and lipid rich microalgal cells (biomass) or droplets (oil). Based on the polar paradox, non-polar antioxidants are therefore predicted to be more effective in these purees. To test this theory the polarity of the different antioxidants was calculated.

As a measure for polarity, the octanol-water partition coefficient, log(POW), of the different antioxidants present in the enriched purees was theoretically investigated by MedChem Designer 3.0 Software

(Simulations Plus, Lancaster, USA). Table 8.1 shows the log(POW) values for the main antioxidants observed in the vegetable purees and microalgal species. In this PhD only the total amount of phenolic compounds was determined. Phenolic compounds typically detected in microalgae and vegetable purees were therefore selected based on literature [222,250,251].

Table 8.1. *The octanol-water partition coefficient of different antioxidants*

Antioxidant compound	Log (POW)
Ascorbic acid	-1.64
Phenolic compounds	
Simple phenols	
Gallic acid	0.68
Ferullic acid	1.54
Coumaric acid	1.68
Salicylic acid	2.14
Flavonoids	
Quercetin	2.01
Luteolin	2.51
Chrysin	3.13
Carotenoids	
Xanthophylls	
Fucoxanthin	7.67
Zeaxanthin	9.32
Diatoxanthin	9.31
Lutein	9.36
Antheraxanthin	9.40
Carotenes	
Lycopene	10.70
α-carotene	11.58
β-carotene	11.62
Tocopherols	
α-tocopherol	10.70

The lower the log(POW) value, the more polar a compound is. Ascorbic acid is the compound with the lowest log(POW) value, followed by phenolic compounds, xanthophylls, and finally tocopherols and carotenes. The main antioxidant capacity was attributed to the carotenoids, which have higher log(POW) values compared to ascorbic

acid and phenolic compounds. This confirmed the polar paradox that non-polar antioxidants are more effective in a polar environment.

Within the carotenoids, xanthophylls have lower log(POW) values compared to carotenes, while primarily the xanthophylls improved the oxidative stability. Additionally, a similar antioxidant capacity of carotenes and tocopherols would be expected based on their log(POW) value. These observations are thus in contradiction with the polar paradox theory. In literature, recent results also contradicted the polar paradox theory as non-polar antioxidants were shown to be less active in polar environments [249]. One can also reason that the antioxidant should be located at the primary site of lipid oxidation, which is often the interface, leading to the hypothesis that there should be an optimum in the polarity. Given the above, it is thus hard to predict the antioxidant capacity of different compounds in different food systems based solely on the polarity [252]. Moreover, a highly reactive antioxidant needs to be mobile and diffuse easily to the site of primary lipid oxidation [253].

8.1.4. FROM THEORY TO PRACTICE

Taken all the obtained results together, it can be concluded that especially **photoautotrophic microalgal biomass** is the appropriate source in order to create an n-3 LC-PUFA enriched vegetable puree with a high oxidative stability. Carotenoids derived from the vegetable purees helped to improve the oxidative stability but alone they were still insufficient as the vegetable purees enriched with heterotrophic microalgae or fish oil were prone to oxidation. During this PhD research, *Schizochytrium* was used as the only representative for heterotrophic microalgae that lack a chloroplast [254,255]. To be able to generalize this statement with more confidence other heterotrophic microalgae (e.g. *Crypthecodinium* sp. and *Ulkenia* sp.) should be tested as well.

Additionally, no specific processing strategy was suggested as only a limited impact of different processing intensities was observed. These conclusions could be drawn irrespective of a batch-to-batch variation of

the microalgae and the vegetable puree, as in each of the different chapters a new batch of the same species was used.

Translation of the results obtained in this PhD for vegetable purees enriched with photoautotrophic biomass to the storage conditions of real food products, can be done by the Q_{10} value. According to literature [256,257], the Q_{10} value of oxidation is 2, meaning that a decrease of the temperature with 10°C halves the rate of oxidation. For the vegetable purees this would mean an oxidatively stable product for at least 2 years when stored refrigerated (7°C) or 1 year when stored at room temperature (17°C). It should be noticed that this prediction does not take into account the microbial stability of the product.

While the use of photoautotrophic microalgal biomass (irrespective of cell (wall) disruption) has been recommended as the most appropriate source in terms of oxidative stability, implications for the bioaccessibility of n-3 LC-PUFA should be taken into account as well. Lowering the cell (wall) integrity might promote the liberation of n-3 LC-PUFA [194], especially for microalgae with a though cell (wall). Additional in-house experiments in collaboration with the Laboratory of Food Technology (KULeuven) showed that the efficiency of cell disruption for enhancing n-3 LC-PUFA bioaccessibility was largely dependent on the cell (wall) integrity of the initial *Nannochloropsis* biomass. However, in general the n-3 LC-PUFA bioaccessibility increased by disruption of the *Nannochloropsis* biomass or extraction of the oil. Further research on the factors influencing the bioaccessibility of n-3 LC-PUFA should thus be executed.

8.2. FUTURE PERSPECTIVES

This PhD was the first study with regard to the use of microalgae as an alternative source of n-3 LC-PUFA in order to boost the nutritional value of vegetable-based products. Promising results were obtained, especially for photoautotrophic biomass, in terms of remaining n-3 LC-PUFA concentration and oxidative stability. Based on these results, it was

hypothesized that the endogenous carotenoids derived from the photoautotrophic microalgae ensured the oxidative stability, but the exact mechanism is still unclear. The strong antioxidant capacity of specific carotenoids of the photoautotrophic microalgae and/or their close location to the n-3 LC-PUFA (when using photoautotrophic microalgae, carotenoids and n-3 LC-PUFA are located in the same cell or even cell organelle) may lead to the observed high oxidative stability. Next to the type of carotenoids and their location, the type of lipid (triacylglycerols, phospholipids, etc.) in which the n-3 LC-PUFA are present may have an influence as well.

It would thus be interesting to further explore whether indeed the antioxidant capacity of specific carotenoids of the photoautotrophic microalgae is high and this not only based on their polarity. On the other hand the importance of the closeness of the n-3 LC-PUFA to the antioxidants and the type of lipid in which n-3 LC-PUFA are present should be investigated. To do so, two different approaches are suggested in section 8.2.1. Additionally, there are some challenges left to implement microalgae on the market (section 8.2.2).

8.2.1. WHY ARE PHOTOAUTOTROPHIC MICROALGAE OXIDATIVELY STABLE?

To fill the gap in the current knowledge explaining the high oxidative stability of n-3 LC-PUFA rich photoautotrophic microalgae, two research approaches can be applied: a bottom-up or top-down strategy. The bottom-up approach, implies that research will start on a simple system and will systematically add complexity to come closer to real vegetable purees. An enriched emulsion containing a concentration of 80 mg n-3 LC-PUFA/100 g emulsion is proposed in the simple system (as in the vegetable purees throughout this PhD). Figure 8.1 represents a scheme of the preparation process.

N-3 LC-PUFA, in the form of phospho-, glycolipids, triacylglycerols or free fatty acids, will be combined with one of the four types of carotenoid at a time. Four different carotenoids, two xanthophylls (zeaxanthin and fucoxanthin) typically found in microalgae and two carotenes typically

found in vegetable purees (lycopene and β-carotene), will be used. For each n-3 LC-PUFA – carotenoid combination, two different preparations will be made: For type 1 the n-3 LC-PUFA rich emulsion is first made and the carotenoids (as powder) are added in a subsequent step, while for type 2 the n-3 LC-PUFA and carotenoid (as powder) are first mixed and this mixture is then added to the aqueous system. Both enriched emulsions are high pressure homogenized in a subsequent step. Based on thermodynamic principles, an equilibrium state is expected which should be reached from both starting conditions. Kinetic limitations may however alter this, which allows to investigate the impact of the carotenoid location. In a later stage, antioxidants can be added as mixtures (instead of as individual compounds) to investigate synergistic effects.

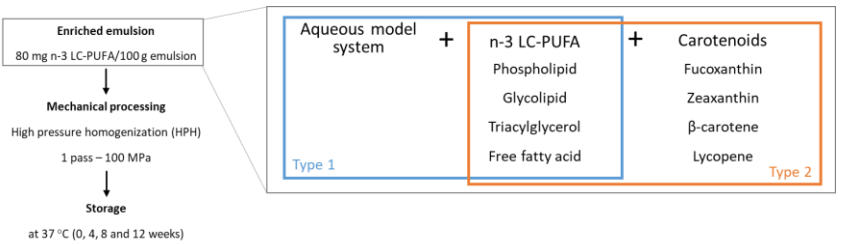


Figure 8.1. *Schematic overview of the preparation process of enriched emulsions via the bottom-up approach.*

In the top-down approach, a model emulsion or suspension (as in chapter 4), representing acidic products, enriched with different combinations of heterotrophic microalgae (oil or biomass) as the supplier of n-3 LC-PUFA and photoautotrophic microalgae (oil or biomass), as the supplier of antioxidants and n-3 LC-PUFA, will be prepared in order to reach a concentration of 80 mg n-3 LC-PUFA/100 g emulsion or suspension. The different combinations lead to the incorporation of different types of lipids in which n-3 LC-PUFA are present, different types of antioxidants and different locations of n-3 LC-PUFA and antioxidants relative to each other. Figure 8.2 presents a scheme of the preparation process according to the top-down approach. For each *Schizochytrium* – *Nannochloropsis* combination, two types of preparations are prepared as described for the bottom-up approach. The use of photoautotrophic oil implies that the

carotenoids are accessible and could thus act as antioxidant for n-3 LC-PUFA from heterotrophic microalgae. Difficulties in transportation through the aqueous phase can however alter this. When photoautotrophic biomass is used, the accessibility of the carotenoids may be limited. In this suggested approach, only antioxidants derived from the photoautotrophic microalgae are present. If they can maintain the oxidative stability of the enriched emulsions/suspensions, irrespective of the delivery form and preparation form, the type of carotenoid is crucial as the antioxidants of the vegetable purees when enriched with *Schizochytrium* were not able to do so.

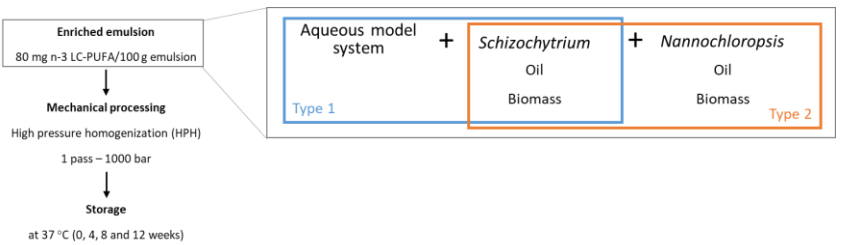


Figure 8.2. *Schematic overview of the preparation process of enriched emulsions via the top-down approach.*

In this PhD, the used microalgal species were cultivated according to their primary energy metabolism: *Isochrysis, Nannochloropsis, Phaeodactylum and Porphyridium* were cultivated photoautotrophically and *Schizochytrium* was cultivated heterotrophically [258]. This implied that the photoautotrophically cultivated microalgae contained a chloroplast, which included photosynthetic pigments, while the heterotrophically cultivated microalgae lacked a chloroplast. Heterotrophic growth of photoautotrophic microalgae is sometimes possible, although it is much slower [54,259]. From a scientific point of view, it could however be interesting to investigate the impact on the oxidative stability of a photoautotrophic microalgal species (which originally contains a chloroplast) cultivated photoautotrophically, heterotrophically or mixotrophically.

Research on the incorporation of n-3 LC-PUFA rich microalgae should also target a broader range of food products. Besides food products with an

antioxidant capacity (vegetable purees), the incorporation of n-3 LC-PUFA rich microalgae in products with pro-oxidative properties (meat products) can be investigated. This will give more insight whether the antioxidant capacity of photoautotrophic microalgae is sufficient to maintain the n-3 LC-PUFA oxidatively stable even in a pro-oxidative environment.

Besides the focus on n-3 LC-PUFA, photoautotrophic microalgae can be applied as a source of interesting antioxidants. The antioxidant potential of microalgae (biomass or extracts) should be further investigated in the view of alternative and natural antioxidants in food products. This should be performed with an accelerated oxidation test at 37°C and not with commonly used methods like FRAP and TEAC to measure the antioxidant potential. The latter methods have shown a weak correlation with the oxidative stability. Furthermore, more research on biorefinery of microalgal biomass towards single and purified compounds must be performed.

8.2.2. CHALLENGES TOWARDS MICROALGAE AS OUR FUTURE FOOD

One of the major challenges for the incorporation of microalgae into our food products is the fact that none of the n-3 LC-PUFA rich photoautotrophic microalgal species has been accepted or authorized under the European novel food regulation. Some applications have however been submitted [9].

Additionally, incorporation of microalgae in a food product can change the consumer acceptance, amongst others in terms of taste, smell, texture and color. Small incorporation concentrations of photoautotrophic microalgae will already alter the color of the food product drastically into green, blue, brown or red, depending on the species used. Therefore, sensorial tests should be executed on the final product enriched with microalgae. At this moment, the European legislation does however not allow taste experiments with n-3 LC-PUFA microalgal biomass, as toxicological safety of novel ingredients and their levels of use must be assessed before any consumption (even for informal

in-house tasting trials) [260]. Sensorial tests without consumption on the other hand are allowed.

Before industrial implementation of photoautotrophic microalgae in food products is possible, a sufficiently large and steady supply of biomass at a reasonable cost is needed. The increase of the ingredient cost of 100 g vegetable puree in order to reach the claim 'rich in n-3 LC-PUFA' by the incorporation of microalgae is shown in Table 8.2. The calculation of the current increase in ingredient price is based on the prices that were paid throughout this PhD. Incorporation of *Schizochytrium*, with a cost of €5/kg dry biomass, does not lead to a drastic increase of the ingredient cost (less than €0.01/100 g vegetable puree). On the other hand, incorporation of photoautotrophic microalgae, with a cost of €240-500/kg dry biomass, leads to an additional cost of €0.8-3.3/100 g vegetable puree depending on the species and incorporation concentration. For comparison, the current price of fruit and vegetable-based products in the supermarket (smoothie, *passata* and pasta sauce) varies between €0.5-1, €0.1-0.5 and €0.2-2/100 g product respectively. Taking into account that the endogenous antioxidants from the photoautotrophic microalgae eliminate the need and thus the cost of extra antioxidants, the cost of incorporation of photoautotrophic microalgae is allowed to be higher compared to heterotrophic microalgae. But even then the current additional cost for the incorporation of photoautotrophic microalgae is clearly too high.

An achievable reduction in production cost for microalgae on a commercial scale under the most suitable conditions of location and production system was predicted by Ruiz *et al.* [7] via a techno-economic model. The production cost for a 100 ha scale production facility in South Spain is predicted at €3.4/kg dry biomass for photoautotrophic microalgae [7]. To obtain a comparable biomass capacity of heterotrophic microalgae a production cost of €4.38/kg dry biomass was predicted [8]. Further optimization of the cultivation in terms of photosynthetic efficiency, biomass concentration, energy use, separation of the biomass from the medium etc., will reduce the predicted production cost of

photoautotrophic and heterotrophic biomass to €1.1 and €1.4/kg dry biomass respectively. Based on these predicted prices, an increase of ingredient cost of less than €0.01/100 g vegetable puree is expected [8]. Currently, the production of n-3 LC-PUFA rich photoautotrophic microalgal biomass is a niche market without competition, which forces the prices. This explains the gap between the predicted cost and the current retail prices.

Based on these perspectives, incorporation of n-3 LC-PUFA rich microalgal biomass could compete with the common n-3 LC-PUFA sources and even with unenriched variants of the vegetable-based products. Additionally, the use of n-3 LC-PUFA rich photoautotrophic microalgae maintains the oxidative stability, while the current sources do not.

Table 8.2. Indication of the increase in current and predicted production cost by the incorporation of microalgal biomass.

	Incorporation concentration (g/100 g vegetable puree)	**n-3 LC-PUFA concentration** (mg/100 g vegetable puree)	**Price of microalgal biomass *** (€/kg dry biomass)	**Additional cost vegetable puree** (€/100 g vegetable puree)	**Predicted price microalgal biomass** [7,8] (€/kg dry biomass)	**Predicted additional cost vegetable puree** (€/100 g vegetable puree)
Isochrysis	6.6	80	500	3.3	1.1	<0.01
Nannochloropsis	2.1	80	380	0.8	1.1	<0.01
Phaeodactylum	3.7	80	240	0.9	1.1	<0.01
Schizochytrium	0.26	80	5	<0.01	1.4	<0.01

** Prices paid in this PhD*

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CURRICULUM VITAE

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EDUCATION

Current	PhD researcher in Bioscience Engineering
	Microbial and Molecular Systems, Food & Lipids Lab, KU Leuven Kulak, Kortrijk, Belgium
	Topic: Oxidative stability of omega-3 long chain poly-unsaturated fatty acids in vegetable purees enriched with microalgae
	Supervisor: Prof. dr. ir. Imogen Foubert
2014	Master in Bioscience Engineering
	KU Leuven, Belgium
	Master thesis: Effect of different lecithins on the crystallization behavior of cocoa butter (Food & lipids lab, KU Leuven Kulak)
2012	Bachelor in Bioscience Engineering
	KU Leuven, Belgium
2009	High school degree, science-mathematics
	Pleinschool, Kortrijk, Belgium

WORK EXPERIENCE

Research experience:

2014 – present	PhD project Laboratory Food & Lipids KU Leuven Kulak, Belgium

Participated at several international conferences with posters and lectures (see list of publications)

Winner of the poster prize at the International symposium on lipid oxidation and antioxidants (Porto, Portugal, 5 - 7 June 2016).

Educational experience:

2014 – 2018	Practical courses Chemical Thermodynamics (2nd bachelor Bioscience Engineering, Chemistry and 3th bachelor Biochemistry)
2014, 2016 & 2018	Practical courses Bio-Organic Chemistry (1st bachelor Bioscience Engineering, Chemistry, Biology, Biochemistry and Pharmacy)
2017 – 2018	Practical courses Transport Phenomena (2nd bachelor Bioscience Engineering, Biology and 3th bachelor Chemistry)
2015 – 2019	Supervisor Project work (2nd bachelor Bioscience engineering)
2016	Supervisor internship Emile Merlevede (2nd bachelor Agro- and Biotechnology)
2016 – 2017	Supervisor master thesis Nele Lagae (1st master Science in Engineering Technology)
2017 – 2018	Supervisor master thesis Robbe Demets (2nd master Bioscience Engineering)
2017 – 2018	Supervisor master thesis Nicky Durnez (2nd master Bioscience Engineering)

SKILLS

Languages

Dutch	Native speaker
English	Very good
French	Good

PC

MS Office (Word, Excel, Powerpoint)

Sigmaplot, JMP

Other skills

Chromatography (GC, LC)

LIST OF PUBLICATIONS

PUBLICATIONS IN INTERNATIONAL PEER-REVIEWED JOURNALS

Bernaerts T.M.M., Verstreken H., **Gheysen L.**, Foubert I., Grauwet T., Van Loey A.M. (2019). The role of cell integrity in the lipid digestibility and in vitro bioaccessibility of ω3-LC-PUFA and carotenoids in *Nannochloropsis* sp. In preparation.

Gheysen L., Durnez N., Devaere J., Bernaerts T., Van Loey A., De Cooman L., Foubert I. (2019). Impact of type of vegetable puree on the oxidative stability of purees enriched with n-3 LC-PUFA rich microalgae, submitted.

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Bernaerts T.M.M., **Gheysen L.**, Foubert I., Hendrickx M.E., Van Loey A.M. (2019). The potential of biopolymers of microalgae as structuring ingredients in food: a review. *Biotechnology Advances*, under review.

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: 1–14.

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