CHALMERS
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Extraction of a seaweed lipid fraction and evaluation of its ability to prevent lipid peroxidation in fish oil

Master's thesis in Biology and Biological Engineering

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CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2017

THESIS FOR THE DEGREE OF MASTER OF SCIENCE

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Cover:
Picture of *Porphyra umbilicalis* (left) and *Ulva lactuca* (right). Collected in Heligoland, Germany 1989-08-08 and 1985-09-10, and gathered from *commons.wikimedia.org*

ACKNOWLEDGEMENTS

I would like to express my gratitude to Ingrid Undeland, my examiner and co-supervisor, for creating this project and giving me the privilege to be a part of designing the project according to personal preferences. Thanks to my supervisors Cecilia Tullberg and Hanna Harrysson for sharing their expertise and experience working with oils and seaweed. All the help and knowledge from the three of you constitutes the foundation of this project.

An additional thanks is dedicated to Nils-Gunnar Carlsson who as a research engineer introduced me to the machines in the lab, and made sure they were running flawless.

Thanks to all people at Food and Nutrition Science for the hospitality and friendliness both in the labs and during lunch/fika. It was easy to feel like a part of the group.

Marcus Hanaeus, Gothenburg 2017

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ABSTRACT

This project is a part of the *Sweaweed* project which aims to evaluate the use of the seaweed species *Porphyra umbilicalis* and *Ulva lactuca* grown along the west coast of Sweden, for high value products. This particular project evaluated the use of the seaweed as a source of long chained n-3 polyunsaturated fatty acids (LC n-3 PUFA), and as a source of natural antioxidants able to preserve other LC n-3 PUFA from oxidation. In a first part of the project, different extraction techniques (polytron, sonication and bead beating) were evaluated for their efficiency in extracting lipophilic compounds (carotenoids, chlorophyll and phenolic compounds) from seaweed into sunflower oil. In the second part, lipid oxidation in seaweed-fortified fish oil was studied during storage as well as during *in vitro* gastrointestinal digestion.

By using a polytron, the highest amounts of carotenoids, chlorophyll and phenolic compounds were extracted from the seaweed into sunflower oil and fish oil. No fortification of LC n-3 PUFAs was however detected in the sunflower oil fortified with *Porphyra umbilicalis* or *Ulva lactuca*. The fish oil fortified with *Porphyra umbilicalis* experienced 29% less degradation of both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) compared to pure fish oil during storage in room temperature and daylight for 28 days. The fish oil fortified with *Ulva lactuca* experienced 22% and 37% less degradation of EPA and DHA compared to pure fish oil. The amount of peroxides after 28 days of storage was 22% and 21% less in the fish oils fortified with *Porphyra umbilicalis* and *Ulva lactuca*. The amount of malondialdehyde (MDA) produced was 40% and 45% less in the fish oil fortified with *Porphyra umbilicalis* and 70% and 68% less in the fish oil fortified with *Ulva lactuca* after 7 and 28 days of storage compared to pure fish oil. The amount of 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) was higher in the fish oil fortified with *Porphyra umbilicalis* after 7 days, and higher in the fish oil fortified with *Ulva lactuca* after 28 days, when compared to pure fish oil. No significant difference was noticed regarding rancid odor in the different fish oils. The extracted seaweed compounds had no preserving effect against oxidation of fish oil during *in vitro* digestion.

Altogether, the fortifying and stabilizing effects from extracting seaweed with food oils were lower than expected, which could be due e.g. to LC n-3 PUFA being firmly bound in lipid classes that are difficult to extract with such a hydrophobic media as oil. Also, simultaneous extraction of pro-oxidative trace elements with the antioxidants may have had a counteracting effect on the stability.

ABBREVIATIONS

AA – Arachidonic acid
ALA – α-linolenic acid
CAT – Catalase
CVD – Cardiovascular disease
DHA – Docosahexaenoic acid
DNPH – 2,4-dinitrophenylhydrazine
DW – Dry weight
EFSA – European Food Safety Authority
EPA – Eicosapentaenoic acid
FA – Fatty acids
GC/MS – Gas chromatography-mass spectrometry
GOED – Global Organization for EPA and DHA
HHE – 4-hydroxy-2-hexenal
HNE – 4-hydroxy-2-nonenal
LA – Linoleic acid
LC – Long chained
LC/MS – Liquid chromatography-mass spectrometry
MDA – Malondialdehyde
n-3 – Omega-3
n-6 – Omega-6
PUFA – Poly unsaturated fatty acids
PV – Peroxide value
ROS – Reactive oxygen species
RPM – Rounds per minute
SC – Short chained
SGF – Simulated gastric fluid
SIF – Simulated intestinal fluid
SOD – Superoxide dismutase
SSF – Simulated salivary fluid

Keywords: Antioxidants, carotenoids, chlorophyll, fatty acids, fish oil, LC n-3 PUFA, macro algae, omega-3, phenolic compounds, seaweed, sunflower oil, *Sweaweed*

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1 INTRODUCTION

As a part of the *Sweaweed* project; this master's thesis project aims to answer whether beneficial fatty acids and lipophilic antioxidants, naturally occurring in the seaweed species *Porphyra umbilicalis* and *Ulva lactuca,* can be extracted and used to fortify and stabilize food grade oils. Hereafter the seaweed species are referred to only by their genus: *Porphyra* and *Ulva*.

1.1 BACKGROUND

Today, many people in the western hemisphere experience a shortage of beneficial n-3 fatty acids (long chain n-3 polyunsaturated fatty acids; LC n-3 PUFA), as westernized foods are rich in n-6 fatty acids. This is discussed as a contributing reason for cardiovascular diseases being the world wide leading cause of death [1]. LC n-3 PUFAs are commonly found in marine foods, such as fatty fish (e.g. herring and mackerel). As fish in the oceans are on the verge of being overfished, exhausting the eco system, it is of interest to come up with alternative sources to LC n-3 PUFA. As LC n-3 PUFA are originally produced in algae and first later in the food chain accumulated in fish, there are possibilities to reduce our dependence on fish from the oceans. If algae are better explored, algae can be cultivated for extraction of valuable nutrients, such as LC n-3 PUFA, or used to feed fish in aquaculture. PUFA however easily goes rancid if not stored in proper conditions together with antioxidants. Algae naturally contain antioxidants which might be able to preserve the beneficial LC n-3 PUFA and other PUFA against oxidation.

1.2 PROJECT DESCRIPTION

Firstly, different physical extraction methods were tested on their ability to extract pigments and phenolic compounds from seaweed into sunflower oil. The color change of the fortified oils was analyzed as a response in these trials. These measurements were used in factorial design to optimize the settings for each method. Sunflower oil was chosen due to its naturally low content of LC n-3 PUFAs, making it possible to determine with higher probability that the LC n-3 PUFAs in fortified oils arise from the seaweed lipid fraction [2]. The method that extracted the highest amount of pigments and phenolic compounds was chosen. The fortified oil processed from this method was analyzed by gas chromatography-mass spectrometry (GC/MS) to evaluate whether the oil had been enriched also with LC n-3 PUFAs. Secondly, to test whether the seaweed can be used as a sustainable and novel natural source of antioxidants preserving LC n-3 PUFAs in fish oil, the best extraction method was integrated into a storage study where pure and fortified fish oil were stored under daylight and dark condition in room temperature (20˚C) and dark in fridge (8˚C). The degradation of fatty acids and the concentration of primary and secondary oxidation products were then quantified. An *in vitro* digestion study was finally performed on pure and fortified fish oils to estimate the level of peroxidation occurring in the stomach and intestine during digestion.

1.3 AIM

The project aimed to answer if it was possible to fortify sunflower oil with LC n-3 PUFAs and antioxidants from *Porphyra* (red seaweed) and *Ulva* (green seaweed) and if so, to quantify the content of these two compound groups. Furthermore, the project examined whether fortification of fish oil with seaweed lipophilic antioxidants decreased lipid peroxidation during storage and *in vitro* digestion.

1.4 HYPOTHESIS

"Seaweed can enrich vegetable oils with LC n-3 PUFAs"

"Seaweed antioxidants have a preservative effect on fish oil"

1.5 LIMITATIONS

- There will be no experimental work regarding fortification of other food with LC n-3 PUFA and antioxidants from seaweed. Experimental work will be limited to fortification of sunflower- and fish oil.

- Only antioxidants extracted with the seaweed lipid fractions will be examined. Furthermore, they will only be evaluated based on their ability to prevent lipid peroxidation in fish oil.

- Subsequently mentioned methods will be solely tested for extraction of a seaweed lipid fraction.

- *In vitro* digestions will only be static, not dynamic.

2 THEORY

This section aims to introduce seaweed and fats (part 2.1-2.3), to then explain the science behind fatty acids and antioxidants (part 2.4-2.7) as this constitutes the foundation of the project.

2.1 ALGAE AND SEAWEED

Algae are eukaryote organisms harvesting sunlight, carbon dioxide and nutrients naturally occurring in the ocean, producing ~70% of our planet's atmospheric oxygen [3]. There are unicellular (microalgae) and multicellular (macroalgae) algae organisms. Macroalgae are commonly referred to as seaweed, easily found along the coastline being either red, green or brown. They anchor themselves to rocks, constituting a habitable environment for smaller aquatic animals. From a biomass point of view, there are advantages of growing seaweed compared to land-based biomass production. Examples being no need for fertilizers and no competition for valuable area on land. As seaweed extracts nutrients from the ocean and bind carbon dioxide and nitrogen, they have a positive impact on the environment [4]. Furthermore seaweeds grow fast and contain various unexplored biomolecules and several important nutrients, making seaweed an attractive biomass for food ingredients, chemicals and other bio-based materials. These advantages have led to seaweed farming being the fastest growing area in aquafarming globally. However, even though Sweden has a vast coast line, its aquafarming is largely undeveloped [5].

2.2 THE *SWEAWEED* PROJECT

Sweaweed is a five-year project funded by the Swedish Foundation for Strategic Research (SSF) in 2015. Together with seven other research projects it is a part of SSF's research program *Biological production systems*. All projects aim to promote industrial processes that can be a part of Sweden's future sustainable bio-based economy. *Sweaweed* is an interdisciplinary collaboration between Chalmers University of Technology, the University of Gothenburg (GU) and the Royal Institute of Technology (KTH). The *Sweaweed* team is divided in five work packages. The first aims to optimize farming and breeding conditions for the red and green seaweed *Porphyra* and *Ulva*, **Figure 1**, naturally growing along the west coast of Sweden. The second work package aims to make protocols for the disintegration of biomass. The third to fifth work package aims to isolate food ingredients, extract fine chemicals and bio-based materials [5].

Figure 1. *Picture of Porphyra (red) and Ulva (green). Collected in Heligoland, Germany 1989-08-08 and 1985-09-10, and gathered from commons.wikimedia.org*

2.3 LIPIDS

Triglycerides, being the most common type of lipid class in oils, have a glycerol backbone connected to three fatty acids, **Figure 2**. When consumed, enzymes in our intestine will degrade the triglyceride into free fatty acids and monoglycerides. Lipids with high amount of saturated fatty acids are commonly solid in room temperature, hence called fats. Lipids with high amount of monounsaturated or polyunsaturated fatty acids (one or more double bonds respectively) leads to the lipids commonly being liquid in room temperature, hence called oils.

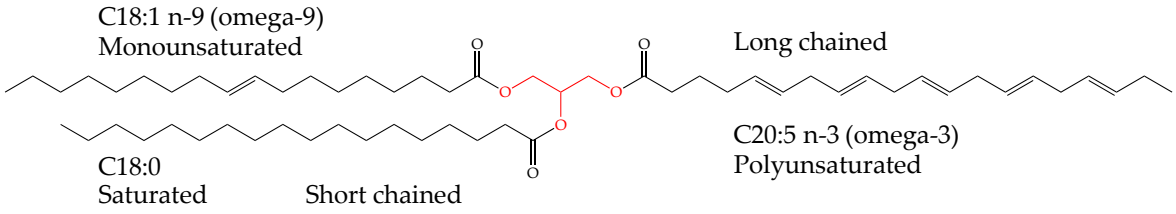

Figure 2. *An example of a triglyceride where the glycerol backbone is marked in red and the three fatty acids in black. Created in ChemDraw Professional 15.0*

2.4 Fatty acids in the body

Unsaturated fatty acids, especially long chained polyunsaturated fatty acids (LC-PUFAs), are more health promoting compared to saturated fatty acids. Consumption of LC-PUFAs have gained much publicity due to their effect in suppressing the pathogenesis of cardiovascular diseases (CVDs), autoimmune diseases, cancer, and inflammatory diseases [6]. To promote beneficial effects for human health, the position of the first double bond on the LC-PUFA is of utmost importance. Depending on if the first double bond is positioned on the third or sixth carbon on the aliphatic chain, counting from the methyl group, the LC-PUFA will be given the nomenclature of omega-3 (n-3) or omega-6 (n-6) respectively. The beneficial health effects are especially emphasized in the LC n-3 PUFA eicosapentaenoic acid (EPA, 20:5) and its elongated and desaturated form docosahexaenoic acid (DHA, 22:6) [7], the latter being important for e.g. normal brain function [8]. LC-PUFAs are oxidized in the body, leading to synthesis of signaling molecules called eicosanoids [9]. Eicosanoids from EPA possesses anti-inflammatory properties, while eicosanoids derived from the LC n-6 PUFA arachidonic acid (AA, 20:4) possesses pro-inflammatory properties [9]. It has been suggested that the greatest positive effect for human health is achieved when the ratio of n-6 to n-3 is balanced, close to 1-2:1 [10]. It is believed that humans evolved on a diet where this ratio was satisfied [10]. However, the modern western diet is often lacking n-3 in favor to n-6, leading to the ratio going as high as 20-25:1 [10]. The human body can itself convert the essential short chained (SC) n-3 C_{18}-PUFA α-linolenic acid (ALA, 18:3) to the beneficial EPA and DHA [11]. However, both ALA and the essential n-6 C_{18}-PUFA linoleic acid (LA, 18:2) is competing over the same enzyme, elongating and desaturating their aliphatic tails [11]. Having higher levels of n-6 in the diet occupies the enzymes, preventing them to convert ALA to EPA/DHA [7]. It is described that the conversion of ALA to EPA/DHA can be as low as 0.2-15% in humans [12]. Due to this, an increased content of LC n-3 PUFAs in food is desired [9]. The European Food Safety Authority (EFSA) consider that an adequate intake for an adult consist of 0.5% ALA, in terms of the daily energy intake, and that 250mg EPA + DHA is consumed [13].

2.5 Sources of LC n-3 PUFA

The current source of LC n-3 PUFAs are derived primarily from wild-caught pelagic fish [14]. The fish is either used to process fish oil, or to feed salmons in aquaculture. The resource is far from sustainable as fish populations in our oceans are on the verge of being overfished [14]. Wild-caught fish are also sensitive towards pollutions, which leads to requirements for high control and careful processing of the fish oil before reaching the common public [12]. To be able to feed a growing population with high quality food, a more sustainable source of LC n-3 PUFA is needed. This as the demand for fish oil is estimated to reach the maximum production rate from wild-caught fish by the year of 2017 according to the Global Organization for EPA and DHA (GOED) [15]. By this time the fish oil derived from wild-caught fish can no longer sustain the growing fish oil industry [15]. As fish itself is not the primary producer of the LC n-3

PUFAs, algae are found to be the source further down the food chain [16]. As LC n-3 PUFAs are consumed, they accumulate in larger concentrations through the food chain [16]. Algae are easy to cultivate as their requirements are limited to sunlight and nutrients naturally occurring in the oceans [16]. Algae are often divided into micro- and macroalgae (seaweed), the former which can accumulate up to ~50% fat on a dry weight basis while the latter contain 1-7% of the dry weight, which makes extraction more difficult [17]. However, the fat content depends both on specie and seasonal change, meaning that there is room for optimization in the cultivation of seaweed as a source of LC n-3 PUFA [17]. There is also room for optimization when it comes to more effective extraction methods being applicable to raw materials with relatively low fat content. Biosynthesis of LC n-3 PUFAs in microalgae is already carried out on a commercial scale, and research strive to increase the production of high value microalgae oils further [18]. Regarding seaweed, research on fat accumulation and extraction is quite limited and needs further attention. Fortifying foods with marine LC n-3 PUFAs EPA and DHA can lead to the production of so called functional foods as the PUFAs provide the food with increased nutritional value which can be the basis for functional health claims [19]. A prerequisite for successful production of functional food containing LC n-3 PUFA, is to ensure the stability of the PUFAs [20].

2.6 LIPID PEROXIDATION

Oxidation of fatty acids (lipid peroxidation) is especially prominent in foods containing PUFAs as they consist of many carbon-carbon double bonds [21]. It leads to not only shorter shelf life and unpleasant taste and odor changes of the food product, but also loss of aforementioned nutritional value [22]. The change in taste and odor is caused e.g. by the production of short chained aldehydes with high volatility. Some of the aldehydes such as malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) also have mutagenic and carcinogenic properties [23]. MDA is a general aldehyde produced during lipid peroxidation, whereas HHE and HNE is specifically synthesized from n-3 and n-6 PUFA respectively [24]. The oxidation process is induced either by enzymes (e.g. lipoxygenase) [25], prooxidants such as iron and copper, or reactive oxygen species (ROS) formed by light (photooxidation) or metabolic processes in oxygenated environments [26]. An example of ROS mediated by light is the excited state of molecular oxygen called singlet oxygen. In this molecule, one of the two valence electrons have changed its spin to achieve the opposite compared to its neighbor, creating a short-lived molecule which quickly stabilize by pairing the two electrons, creating a long-lived singlet oxygen. This form of oxygen is linked to peroxidation of fatty acids [27]. Other common ROS are radicals such as the hydroxyl (·OH), hydroperoxyl (HO_2·) and su*PEROXIDE* (O_2^-·). Superoxide can together with hydrogen peroxide (H_2O_2) undergo the Haber-Weiss reaction, catalyzed by ferric/ferrous (Fe^{3+}/Fe^{2+}) ions, to create hydroxide (·OH), hydroxyl and singlet oxygen [28].

For a schematic example of lipid peroxidation, see **Figure 3.** Reaction **(1)** in **Figure 3** shows ·OH scavenging a hydrogen from a fatty acid (LH) [29]. This reaction yields a lipid radical (L·) reacting with molecular oxygen (O_2), **(2) Figure 3.** The peroxyl radical (LOO·) created reacts with other fatty acids, **(3) Figure 3.** This reaction yields another lipid radical and lipid hydroperoxide (LOOH), **(4) Figure 3.** Being a primary lipid oxidation product, lipid hydroperoxides can decompose to secondary lipid oxidation products such as short chained aldehydes with strong smell and aforementioned mutagenic and carcinogenic properties. The lipid peroxidation cycle can to some extent terminate itself when two free radicals meet and react with each other.

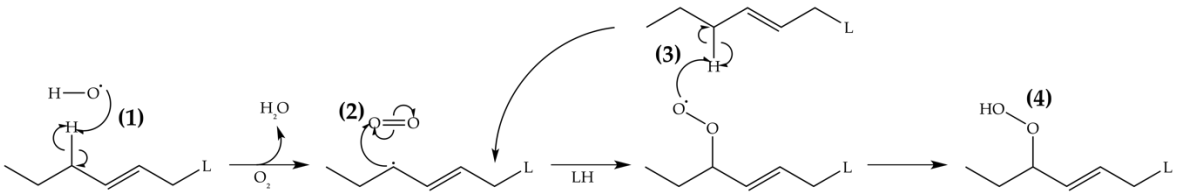

Figure 3. *Schematic overview of lipid peroxidation modified in ChemDraw Professional 15.0 from I.S. Young and J. McEneny (2001) [30]*

Lipid peroxidation not only occurs during storage, but also during digestion of food containing PUFA [31]. Consuming food rich in prooxidants, such as chlorophyll and trace metals found in seaweed, have been shown *in vitro to* lead to a significant (α=0.05) increase in lipid peroxidation in the gastrointestinal tract [32]. In the gastric tract, the oil is still in a droplet but exposed to lipases active at the low pH and high temperature (37˚C). In the intestinal phase the oil is emulsified and exposed to more lipases degrading the triglycerides. The free fatty acids are here highly vulnerable for lipid peroxidation. The production of aldehydes is inevitable. For the intestinal fluid, the lethal concentration 50 (LC50) over a 16 hour period are 600-3000μM MDA and 20-60μM HHE and HNE [33]. The LC50 tells which concentration that induce death in 50% of the endothelial cells [33].

2.7 ANTIOXIDANTS AND PROOXIDANTS

The presence of antioxidants along with the PUFA is crucial in order to reduce the lipid peroxidation to a minimum. Antioxidants do this by inhibit the formation of, or scavenge, ROS or lipid radicals [32]. There are enzymatic antioxidants such as superoxide dismutase (SOD) and catalase (CAT), and non-enzymatic antioxidants such as carotenoids and phenolic compounds [34]. Carotenoids are present in the chloroplast of plant system. During the absorption of light in photosynthesis, chlorophyll is oxidized. If photosynthetic processes cannot cope with the light intensity, the chlorophyll will be oxidized into its triple state working as a photosensitizer to form singlet oxygen. Carotenoids actively quench triple chlorophyll and scavenge singlet oxygen to thereby protect the photosynthetic membrane from oxidation [34]. The content of chlorophylls and carotenoids in oils are therefore of utmost importance. A chlorophyll concentration of 2ppm in purified olive oil

increased the peroxide formation by more than threefold if stored in light conditions [35]. However, in dark condition an increase of chlorophyll did not affect the peroxide formation [35]. In contrast *Fakourelis et al. (1987)* showed that a β-carotene concentration of 20ppm caused half as much peroxide formation as the control when stored in light condition [35]. Another antioxidant feature of carotenoids is the absorption of light between 400-500nm, protecting other molecules from photooxidation [36]. Due to this relationship of chlorophylls and carotenoids, it is wanted to decrease the concentration of chlorophylls and increase the concentration of carotenoids in the fortified oils later in this report.

Phenolic compounds donate a hydrogen atom to radicals to thereby terminate lipid peroxidation and make sure it does not propagate indefinitely [37]. Examples of these are tocopherols/-trienols which donates a hydrogen to lipid peroxyl radical, terminating the peroxidation chain reaction showed in **Figure 3** [38]. In the body, oxidized tocopherols/-trienols can be regenerated through reduction by ascorbic acid to dehydroascorbate [39]. Regeneration of ascorbic acid is possible through the thiol redox cycle generated by GSH reductase using NADPH as a substrate [39].

Synthetically produced antioxidants and commercially available natural antioxidants have shown lower effect than desired in the preservation of some food enriched with LC n-3 PUFAs [20]. Furthermore, synthetically produced antioxidants are restricted due to health risks in humans [40]. In addition to this, many natural antioxidants are extracted from herbs, and the extracts are provided with strong flavor and color which do not always match with the food product. This has led to the search for novel natural antioxidants to retain the nutritional value of food sensitive to oxidation, both during storage and digestion [19]. The presence of LC n-3 PUFAs in seaweed lead to the belief that antioxidants (such as carotenoids and phenolic compounds) naturally found in seaweed can also be used to preserve other LC n-3 PUFAs [19]. It was recently shown that seaweed extracts managed to prevent lipid peroxidation in fish systems with high LC n-3 PUFAs [41].

3 MATERIALS AND METHODS

3.1 CRUDE COMPOSITION OF SEAWEED BIOMASS

Both species of seaweed were collected at Tjärnö, Sweden the 20th of June 2016 and stored in -20˚C until 27th of September 2016 when the seaweed were ground, using a *KitchenAid Artisan 5KSM150* with *Food Grinder Attachment (FGA) 2* and the 5mm hole plate. Half of each biomass was portioned into 50ml *Fisher Scientific* centrifuge tubes and stored in *New Brunswick Scientific Ultra Low Temperature Freezer U535* at -80˚C. The other half was put in a *Heto Drywinner (DW) 6-55* freeze dryer with condensed wall temperature at -54˚C and pressure at 0.054 hPa. After 7 days of freeze drying, the dry biomass was portioned and stored likewise the wet biomass.

3.1.1 DRY WEIGHT

The dry weight of the wet biomass was calculated by drying a triplicate of 5g of wet biomass for each specie, in a 105˚C oven overnight. The dry biomass was then let to cool down in a dry desiccator before weighed.

3.1.2 TOTAL LIPID CONTENT

The lipid content of the seaweed was quantified gravimetrically after performing extraction of total lipids with a method developed by *Lee et al.* (1996) and later modified by *Undeland et al.* (2002). A triplicate of 1g freeze dried biomass from each specie were weighed in 50ml centrifuge tubes where 10ml ice-cold 1:1 mixture of chloroform and methanol were added. A polytron (*Ultra Turrax IKA® T18 basic*) was used at 14000rpm to homogenize the sample. The polytron was thereafter cleaned in another 10ml ice-cold 1:1 mixture of chloroform and methanol. This volume was then added to the tube containing the biomass. A volume of 6.16ml 0.5% NaCl (*Scharlau*) was added whereafter the tubes were vortexted for 30 seconds in a *Scientific Industries Vortex-Genie 2*. The tubes were then centrifuged in a *Heraeus Multifuge 1 S-R* for 6 minutes at 2000g and 4˚C. The lower chloroform phase was transferred to pre-weighed glass tubes by using a glass syringe. The glass tubes were weighed again before left to evaporate in room temperature with a constant flow of N_2-gas. The glass tubes were finally weighed again, yielding the total lipid content of the seaweed.

3.1.3 FATTY ACID COMPOSITION

To determine the fatty acid composition, an in-house method for direct fatty acid methylation published by *Cavonius et al.* (2014) was used [42]. A duplicate of 50mg freeze dried biomass from each species was weighed and put into glass tubes. As internal standard, 100µl 1mg/ml C17:0 in toluene was added to each sample. One ml of toluene, and 1ml of 10% HCl in methanol were added to each tube before incubated for 2 hours at 70˚C. The methylation was stopped by adding 0.2ml mQ-H_2O followed by vortexing. Four ml petroleum ether and 1ml diethyl ether were added whereafter the tubes were vortexted before centrifuged for 6 minutes at 2500g at 4˚C. The organic upper phase was transferred to fresh tubes using a glass syringe. The solvents were then evaporated at 40˚C with a constant flow of N_2. Three ml iso-octane was added to

each tube before injection to an *Agilent 7890* GC system equipped with a *J&W DB*-wax column (30m×0.25mm×0.25µm) and interfaced with an *Agilent 5975 C* triple-axis MS detector in electron impact mode. The injection volume was 1µl with a 15:1 split at an inlet temperature of 275˚C. Helium was used as the carrier gas, with a fixed flowrate of 1ml/min throughout the program. The program started at 100˚C for 0 min to then increase 4˚C/min to 205˚C, to thereafter increase 1˚C/min to 230˚C whereafter the temperature was held for 5 minutes. *GLC Reference Standard: 463* from *Nu-Chek Prep, Inc.* was used as external standard. The data was gathered and analyzed by using the software *MSD ChemSation E.02.01.1177* with minimum peak area set to 300 000 area counts.

3.1.4 PIGMENTS AND PHENOLIC COMPOUNDS

The concentration of pigments and phenolic compounds in the seaweed were extracted according to a method described by *Veide et al.* (2015) [43]. A triplicate of 0.5g of dry *Porphyra* and *Ulva* was extracted in 5ml 80% acetone in 13ml *Fisher Scientific* centrifuge tubes sealed with aluminum foil to protect light sensitive compounds. The tubes were put in a *Heidolph Reax 2* rotary incubator set on 30rpm in room temperature overnight. The tubes were then centrifuged for 5 minutes at 3000g, and the supernatant transferred to 5ml *Fisher Scientific Eppendorf™ tubes*.

3.1.4.1 PIGMENTS

The absorbance of the samples were measured at the wavelengths 663, 647 and 470nm using an *Agilent Technologies Cary 60 UV-Vis* spectrophotometer with a 10mm Quartz cuvette from *Hellma Analytics* [44]. The spectrophotometer was blanked with 80% acetone. The data was gathered by using the *Cary WinUV Scan Application* from *Agilent Technologies*. By using absorption coefficients for leaf pigment extracts in 80% acetone solvent acquired by *Hartmut K. Lichtenthaler* (1987), an estimation of the amount of carotenoids and chlorophyll was calculated according to **Equation (1) – (3)** [44]:

Equation (1) $$C_a = \text{Chlorophyll A} = 12.25A_{663} - 2.79A_{647}$$

Equation (2) $$C_b = \text{Chlorophyll B} = 21.50A_{647} - 5.10A_{663}$$

Equation (3) $$C_{x+c} = \text{Carotenoids} = \frac{1000A_{470} - 1.82C_a - 85.02C_b}{198}$$

The total concentration of chlorophyll was calculated by adding the concentration of chlorophyll A and B from **Equation (1)** and **(2)**. The total concentration of carotenoids was calculated by using **Equation (3)**. The concentration is in the unit [µg pigment/ml extract]; easily converted to [µg pigment/g dry seaweed] (ppm).

3.1.4.2 PHENOLIC COMPOUNDS

An external standard curve had to be made where phloroglucinol was diluted in 80% (v/v) acetone in mQ-H_2O to concentrations ranging between 0-100µg/ml [43]. One ml of the phloroglucinol in acetone was mixed with 500µl 10%(v/v) *Sigma-Aldrich* Folin Ciocalteu reagent in mQ-H_2O [43, 45]. After 5 minutes, 500µl of 7.5%(w/v) reagent grade $NaHCO_3$ in mQ-H_2O was added whereafter the absorbance was measured at 765nm using a 10mm Quartz cuvette [43, 45]. The data was gathered by using the software *Cary WinUV Simple Reads Application* from *Agilent Technologies*. The spectrophotometer was blanked with the aforementioned ratio of 80% acetone, Folin Ciocalteu reagent and $NaHCO_3$. The standard curve can be seen in **Figure A.1** in **Appendix A**.

The concentration of phenolic compounds in the acetone extract from part 3.1.4 was estimated by replacing 1ml of phloroglucinol in acetone with 1ml acetone extract in the aforementioned method. The equation derived from the standard curve was then used to calculate the concentration of phenolic compounds/dry seaweed [µg/g] (ppm).

3.2 EXTRACTION OF SEAWEED INTO SUNFLOWER OIL

Four in house physical extraction equipment's (polytron, beadbeater, sonicator bath and sonicator probe) were examined to facilitate extraction of lipophilic compounds from the seaweed into sunflower oil (*Brökelmann + Co Oelmühle GmbH + Co, Germany*) purchased from the local market (*Coop Konsum, Landala*). When using the polytron, sonicator bath and the sonicator probe, 0.5g of dry weight seaweed biomass in freeze dried or wet form was mixed with 4.5g oil in 13ml centrifuge tubes. Giving a dry biomass to oil weight ratio of 1:9. When using the bead beater, both masses were doubled to cope with the larger 50ml centrifuge tubes needed to run the bead beater. The mixtures were vortexed to make the samples as homogenous as possible. The centrifuge tubes were kept on ice to keep a stable temperature just above 0˚C, before the extraction methods were conducted. After extraction the tubes were centrifuged for 2 minutes at 4000g, to remove the seaweed debris and leave a clean fortified oil as the supernatant. The supernatant was transferred to fresh 5ml Eppendorf tubes, put on ice and analyzed according to part 3.2.2.

3.2.1 OPTIMIZATION OF EXTRACTION METHODS

As physical extraction methods generate heat, which might promote lipid peroxidation, the temperature profile was measured on pure sunflower oil for each of the extraction equipment's in order to set suitable time spans for which the extraction methods would be run. The extraction methods were conducted in a duplicate on separate days. Factorial design (see **Table 1** for a general layout) was applied in order to determine the most optimal settings for each method. Sample number 0 is pure sunflower oil which was treated the same as the fortified oils to be able to compare the effect each extraction method per se had on the seaweed biomass. By using appropriate factorial designs together with replicates, results from analysis of

lipophilic compounds and color were used to create ANOVA-tables, able to estimate which parameters that were of largest importance for each disintegration method. A normal distribution was assumed for all replicates, and the results are reported as means and standard deviation. For all statistical analysis, α was set to 0.05 in a two-tailed normal distribution. The extracted amounts of lipophilic compounds and total change in color for the four extraction equipment's explained in part 3.2.1 can be seen in **Appendix B** together with the statistical data. The general experimental design for fortification of sunflower oil is shown in **Figure 4.**

Table 1. *General 2^4-factorial design used when testing the different equipment's*

-/+	*Porphyra/Ulva*	Dry/Wet	Short/Long	Low/High
	Specie	**State**	**Time**	**Power level**
0	0	0	-	-
1	-	-	-	-
2	+	-	-	-
3	-	+	-	-
4	+	+	-	-
5	-	-	+	-
6	+	-	+	-
7	-	+	+	-
8	+	+	+	-
9	-	-	-	+
10	+	-	-	+
11	-	+	-	+
12	+	+	-	+
13	-	-	+	+
14	+	-	+	+
15	-	+	+	+
16	+	+	+	+

3.2.1.1 POLYTRON

The polytron used was an *Ultra Turrax IKA® T18 basic*. For this study the frequencies of 14000rpm and 24000rpm were tested. It was not possible to measure the temperature continuously, why the temperature profile was constructed by measuring 4 separate runs on each power level. The times of 60 and 120 seconds were chosen as levels in a 2^4-factorial design shown in **Table 2**.

3.2.1.2 BEADBEATER

A *Retsch MM400* beadbeater with washed glass beads (212-300µm) from *Sigma-Aldrich* was used with inserts for 50ml centrifuge tubes. For a normal sample with 1g seaweed (DW) and 9g sunflower oil, 1ml of glass beads were used. The beadbeater did not offer the opportunity to measure the temperature continuously. Therefore the temperature profile was constructed by measuring 6 separate runs on 1200 and 1800 rpm. The times of 60 and 300 seconds were chosen to be the levels in the 2^4-factorial design shown in **Table 3**.

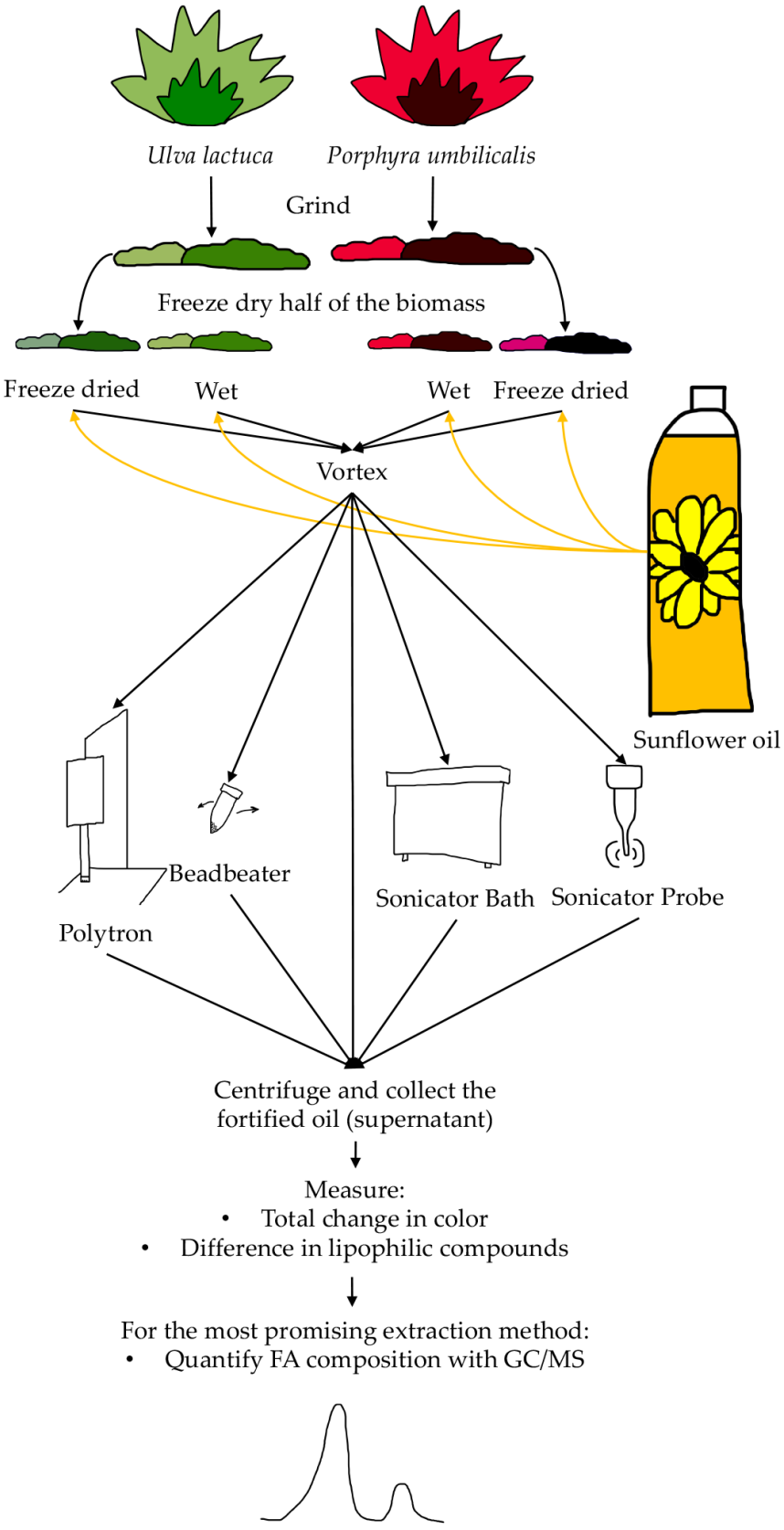

Figure 4. *Approach used for extraction and analysis of fortified sunflower oil. FA = fatty acids. GC/MS = Gas chromatography mass spectrometry.*

Table 2. *The 2^4-factorial design used for evaluation of the polytron*

	Specie	State	Time [min]	Freq. [rpm]
0	0	0	1	14000
1	*Porphyra*	Dry	1	14000
2	*Ulva*	Dry	1	14000
3	*Porphyra*	Wet	1	14000
4	*Ulva*	Wet	1	14000
5	*Porphyra*	Dry	2	14000
6	*Ulva*	Dry	2	14000
7	*Porphyra*	Wet	2	14000
8	*Ulva*	Wet	2	14000
9	*Porphyra*	Dry	1	24000
10	*Ulva*	Dry	1	24000
11	*Porphyra*	Wet	1	24000
12	*Ulva*	Wet	1	24000
13	*Porphyra*	Dry	2	24000
14	*Ulva*	Dry	2	24000
15	*Porphyra*	Wet	2	24000
16	*Ulva*	Wet	2	24000

Table 3. *The 2^4-factorial design used for evaluation of the beadbeater*

	Specie	State	Time [min]	Freq. [rpm]
0	0	0	1	1200
1	*Porphyra*	Dry	1	1200
2	*Ulva*	Dry	1	1200
3	*Porphyra*	Wet	1	1200
4	*Ulva*	Wet	1	1200
5	*Porphyra*	Dry	5	1200
6	*Ulva*	Dry	5	1200
7	*Porphyra*	Wet	5	1200
8	*Ulva*	Wet	5	1200
9	*Porphyra*	Dry	1	1800
10	*Ulva*	Dry	1	1800
11	*Porphyra*	Wet	1	1800
12	*Ulva*	Wet	1	1800
13	*Porphyra*	Dry	5	1800
14	*Ulva*	Dry	5	1800
15	*Porphyra*	Wet	5	1800
16	*Ulva*	Wet	5	1800

3.2.1.3 SONICATOR BATH

An *Elma S15* ultrasonic bath filled with water at ambient temperature was run with 13ml centrifuge tubes inserted in a floating piece of Styrofoam. High frequency sound waves are spread in the water bath, to some extent penetrating the plastic centrifuge tubes and interacting with the sample. The frequency could not be changed for this equipment, so it remained constant at 37kHz. The increase in temperature when using the sonicator bath was measured continuously, starting with the oil being equal to the surrounding water at ~20˚C. The times of 60 and 300 seconds were chosen to be the levels in the 2^3-factorial design shown in **Table 4**.

Table 4. *The 2^3-factorial design used for evaluation of the sonicator bath*

	Specie	State	Time [min]
0	0	0	1
1	*Porphyra*	Dry	1
2	*Ulva*	Dry	1
3	*Porphyra*	Wet	1
4	*Ulva*	Wet	1
5	*Porphyra*	Dry	5
6	*Ulva*	Dry	5
7	*Porphyra*	Wet	5
8	*Ulva*	Wet	5

3.2.1.4 SONICATOR PROBE

A *Branson Sonifier 250* connected to a 3mm ultrasonicator probe was used with the tip of the probe inserted half-way into the sample volume. The probe has similar technology as the previously mentioned sonicator bath, but the soundwaves originate directly in the sample instead of in a surrounding water bath. The sonicator probe has two adjustable settings. The output level controls the frequency of the sonic wave originating from the probe. For a specific sample; the frequency is proportional to the power of the sonication delivered at each pulse. The duration of each pulse is controlled by the second setting; duty cycle.

For creation of the temperature profile, the duty cycle was set to 50% (medium setting), and the output level was chosen to 3 and 6 (medium and high). The temperature was measured continuously with an *Armatherm GTH 1160* digital thermometer as the samples were standing on ice. Due to the rapidly increasing temperature and large variation of settings, it was decided that no specific time levels would be chosen. Instead the ability to continuously measure the temperature decided the time for each different combination of duty cycle and output level. The temperature limit was decided to be set to 30˚C, similar to the temperature reached by most other extraction equipment.

To be able to find an optimum setting for duty cycle and output level, the duty cycle was chosen to range between 20-60% and the output level was chosen to range between 2-6 in a 2^2-factorial design with center points as shown in **Figure 5**. Single replicates were run in the corner points and a triplicate was run for the center point to be able to calculate the error of the method. Only one species of seaweed (*Ulva*) was extracted to give more time to find an optimal setting. Also it was not possible to extract the wet seaweed with the sonicator probe, as it turned into an non-extractable slime during extraction.

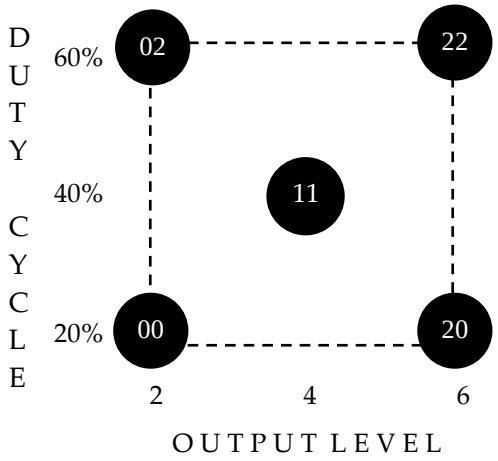

Figure 5. *The 2^2-factorial design used to evaluate the first test of the sonicator probe*

For the second test a 3^2-factorial design with output level 4-6 and a duty cycle between 30-50% was used. The center point was run in triplicate. The experimental design can be seen in **Figure 6**. The third test was run in order to evaluate the duty cycle. The output level was therefore kept constant on 5, as the duty cycle ranged between 40-80%. Each setting was tested in a duplicate. A fourth test was designed to examine whether dry *Porphyra* had a different optimum compared to *Ulva*. Due to that the red seaweed was gelling much more than the green, it had to be disintegrated further in a coffee grinder. This allowed the sonicator probe to come in contact with the oil and extract the seaweed. A 3^2-factorial design was conducted according to **Figure 7**. A triplicate was run on the center point, and single runs on the surrounding points.

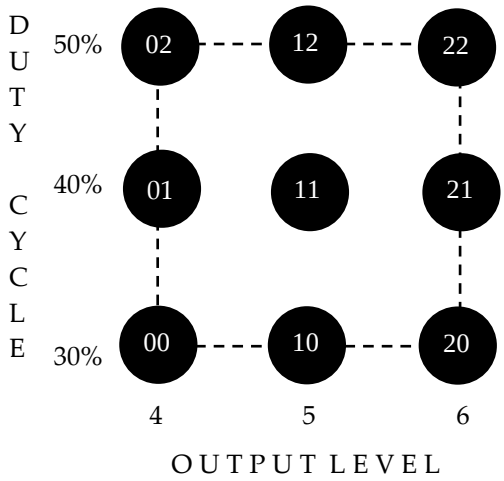

Figure 6. *The 3^2-factorial design used to evaluate the second test of the sonicator probe*

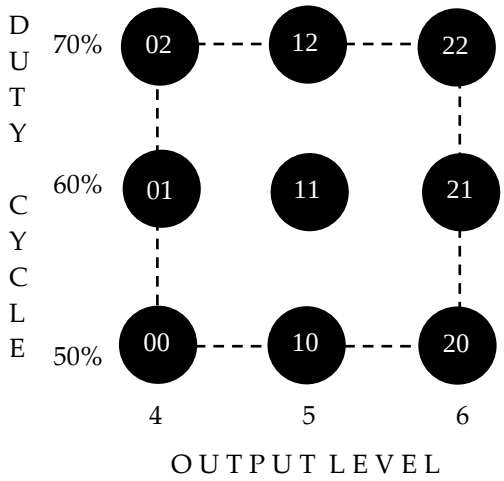

Figure 7. *The 3^2-factorial design used to evaluate the fourth test of the sonicator probe*

3.2.2 Analysis of fortified oils

3.2.2.1 Total change in color

The color of the sunflower oils when fortified with seaweed biomass was analyzed by using a *Minolta Colorimeter CR-400* to measure the total color change on 1ml of oil for all chosen methods. One ml was pipetted into white lids (*Cerbo laboratory lid 30*) from *Nolato*, and the average out of 5 measurements was recorded. The *Minolta Colorimeter* measured the color in the Hunter color measurement system where L* represent lightness from black to white (0-100), a* represents green to red, and b* represents blue to yellow [46]. A total change in color indicates that pigments from the seaweed have been extracted to the oil. The data was gathered by using the software *SpectraMagic NX Color Data Software CM-S100w*.

3.2.2.2 Pigments and phenolic compounds

To measure the concentration of pigments and phenolic compounds, 0.5g of oil was dissolved in 5ml 80% acetone and thereafter analyzed as described in part 3.1.4.

3.2.2.3 Fatty acid composition

The fatty acid composition was measured on the extraction method that was considered most promising. The method to measure the fatty acid composition in the fortified oils is similar to the method described in part 3.1.3, with a few exceptions: Twenty mg of oil was used instead of 50mg freeze dried seaweed. The concentration of the internal standard C17:0 was 10mg/ml instead of 1mg/ml. Finally, twice the volume of iso-octane was used to dilute the samples.

Table 5. *The best setting for each extraction method*

Method	Time/Degrees	Power level/Frequency
Polytron	2 min	24000rpm
Sonicator Probe	30˚C	Output level: 5 Duty cycle: 60%
Beadbeater	5 min	1800 rpm
Sonicator Bath	5 min	-
Soaking in room temperature	20˚C	-

3.2.3 COMPARING THE BEST SETTING OF EACH METHOD

The best settings for each method were evaluated against each other. The effect of having the seaweed soaked in sunflower oil in room temperature and subjecting it to vortex 4 times, was also tried. The best setting for each method can be seen in **Table 5**. To be able to compare the extraction methods, dry biomass was used in all cases. For this experiment, the biomass was further ground in a coffee grinder to achieve a more homogenous powder, and by that more comparable results. A duplicate was run for each method. A two sample t-test assuming equal variance was performed to compare the results between the different methods.

3.2.4 EFFECT OF HEAT ON EXTRACTION

The polytron and sonicator probe were found to be the two most promising extraction methods. To evaluate whether the extraction temperature had any impact on the extractability, the equipment's were used at their optimal settings and run on dry *Porphyra* mixed with sunflower oil until the sample temperature reached 30 and 50˚C. To evaluate the effect of heat on extractability, another set of samples were left in a 100˚C oven until the oil temperature reached 30 and 50˚C. The samples were vortexed vigorously before and after the heat treatment. A duplicate was run for each method and temperature. Once again, a two sample t-test assuming equal variance was performed.

3.2.5 ANALYSIS OF THE MOST PROMISING METHOD

Using the polytron at 24000rpm until 50˚C was reached was the most successful method to extract carotenoids, and gave the largest change in color, see part 4.2.3. The fatty acid composition of the fortified oil by using this method was analyzed. To increase the probability to identify any difference in the fatty acid composition, 0.5g of dry seaweed was extracted in 2g sunflower oil instead of 4.5g sunflower oil as used previously. The polytron was run for 3 minutes at 24000rpm to reach 50˚C. Also a blank with pure sunflower oil was treated the same. A duplicate of each oil was methylated followed by GC/MS analysis according to method described in part 3.2.2.3. A two sample t-test assuming equal variance was performed to detect any differences between the oils.

3.3 EXTRACTION OF SEAWEED INTO FISH OIL

3.3.1 EFFECT OF HEAT ON LIPID PEROXIDATION IN FISH OIL

Using the polytron at 24000rpm, extracting the samples for 7 minutes until the temperature had risen to 50˚C was found to be the most successful extraction method to fortify sunflower oil, see results in part 4.2.3. Since this method involves heating the oil to 50˚C, it was of interest to evaluate the degree of oxidation occurring when exerting fish oil to heat. Cod liver oil (hereafter referred to as fish oil) supplied from *Lysi hf* (Reykjavik, Iceland) with a natural content of 300ppm vitamin A and no added antioxidants was used. The fish oil was thawed and divided into 3 separate Eppendorf tubes. One tube remained on ice whereas the other two were heated to 30 and 50˚C in a 100˚C oven. It took 150 seconds to heat the fish oil to 30˚C, and 7 minutes to reach 50˚C. An analysis of peroxide value (PV) was done according to part 3.3.1.1, to determine the amount of primary oxidation the oil undergoes when heated. A two sample t-test assuming equal variance was executed to detect the differences in PV.

3.3.1.1 PEROXIDE VALUE

Analysis of PV was done according to the method by *Shantha and Decker* (1994), later modified by *Undeland et al.* (2002) [47, 48]. A duplicate of 0.15g of each of the above oils were mixed with 2ml ice-cold 2:1 mixture of chloroform and methanol, whereafter diluted 10 times to a total volume of 2ml. 1.33ml ice-cold 1:1 mixture of chloroform and methanol was added together with 33.4µl ammonium thiocyanate in mQ-H_2O (0.3g/mI). After 2 seconds of vortexing, 33.4µl iron (II) chloride solution was added, followed by another 2 seconds of vortexing. The iron (II) chloride solution was acquired by dissolving $BaCl_2$*$2H_2O$ with 0.4M HCl (0.008g/ml) and $FeSo_4$*$7H_2O$ with mQ-H_2O (0.01g/ml). Equal volumes of these solutions were then vortexed 1 minute, whereafter the upper phase was acquired after centrifuging 3 minutes at 3000g. The absorbance at 500nm was recorded after 20 minutes of dark incubation in room temperature. As blank, a 2:1 mixture of chloroform and methanol was used. A standard curve was made from cumene hydroperoxide (CPO) diluted in a 2:1 mixture of chloroform and methanol to concentrations of 0 to 30µM. The standard curve can be seen in **Figure A.2** in **Appendix A**. The standards were then treated as the fish oil samples in 2:1 mixtures of chloroform and methanol.

3.3.2 FORTIFICATION OF FISH OIL

The aforementioned polytron method was used in order to evaluate whether the extracted antioxidants from the seaweed were able to stabilize the PUFAs in fish oil. A new batch of fish oil supplied from *Lysi hf* (Reykjavik, Iceland) with a natural content of 388ppm vitamin A and no added antioxidants, was acquired. A triplicate of freeze dried and coffee grinded *Porphyra* and *Ulva* was weighed to 4g in each of three 50ml centrifuge tubes. To keep the weight ratio of biomass to oil at 1:9, 36g of fish oil was added to each tube except the tubes containing pure fish oil where 40g of fish oil was added. The samples were vortexed and immediately put on ice before the extraction

method was applied. The pure and fortified fish oil was obtained and used to measure lipophilic compounds and fatty acid composition. Furthermore the oils were used to study the effects of storage- and an *in vitro* gastro intestinal digestion on lipid peroxidation. See **Figure 8** for an overview of the fortification of fish oil.

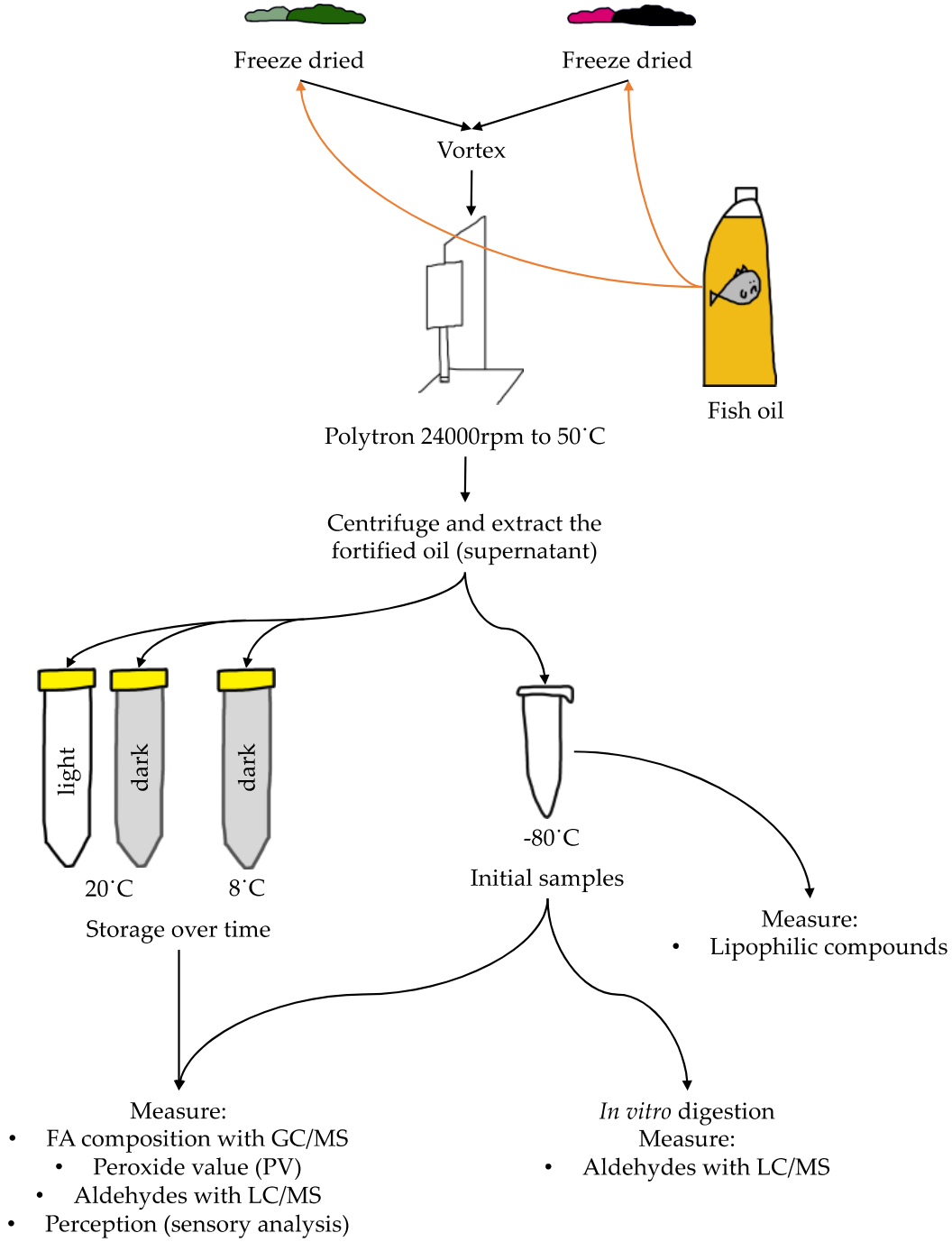

Figure 8. *Approach in the analysis of oxidative stability of fortified fish oils during storage and in vitro digestion*

3.3.2.1 PIGMENTS AND PHENOLIC COMPOUNDS

The pigments and phenolic compounds in the fish oil control and the fortified fish oils were measured as described in part 3.2.2.2.

3.3.3 STORAGE STUDY OF PURE AND FORTIFIED FISH OIL

Nine ml from each of the fish oil control and fortified fish oil was pipetted into 50ml centrifuge tubes. One set was covered in aluminum foil and put in the fridge (8˚C). Another set was covered in aluminum foil and put in room temperature (20˚C). A third set of samples was prepared and put in room temperature where it was exposed to daylight. A sample size of 500µl from each oil was taken after 3 and 7 days, and thereafter at a weekly interval until 6 weeks had passed. Samples were put in 1.5ml *Microcentrifuge Tubes* and flushed with N_2 before stored in -80˚C.

3.3.3.1 CHANGES IN FATTY ACID COMPOSITION

The fatty acid composition of the oils at day of fortification and after 28 days of daylight storage at room temperature was measured with GC/MS as described in part 3.2.2.3. This was preformed to be able to detect the degradation of fatty acids during storage. The result was analyzed with a two sample t-test assuming equal variance. This allowed comparison of the fatty acid patterns between fish oil control and fish oil fortified with *Porphyra* and *Ulva*. Statistical data from the two sample t-test assuming equal variance are shown in **Appendix C**.

3.3.3.2 CHANGES IN PEROXIDE VALUE

Peroxide value was measured on the samples from day 0 and 28 by using the same method as described in part 3.3.1.1. However, the oils were diluted 20 and 200 times for the 0 and 28 day samples respectively.

3.3.3.3 CHANGES IN REACTIVE ALDEHYDES

Malondialdehyde (MDA), 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE) was measured by using the same method as *Tullberg et al.* (2016) [31]. In 1.5ml *Microcentrifuge Tubes*, 80mg of each oil was weighed and mixed with 420µl mQ-H_2O. BHT (0.1g/ml, 4.5M in MeOH) and EDTA (0.02M in mQ-H_2O) was added in volumes of 20µl and 40µl respectively. The tubes were vortexed for 10 seconds and then left in room temperature for 5 minutes before centrifuged 2 minutes at 16000g at 4˚C. The supernatant was transferred to fresh 1.5ml *Microcentrifuge Tubes* whereafter the samples were derivatized with 25µl 2,4-dinitrophenylhydrazine (DNPH) (2mg/ml, 10mM in MeOH). The tubes were vortexed for 10 seconds and left to incubate in room temperature for 60 minutes. The samples were then extracted twice, with 500µl dichloromethane whereafter the samples were vortexed for 10 seconds and centrifuged 2 minutes at 16000g at 4˚C. The lower phases were transferred to new 1.5ml *Microcentrifuge Tubes* and left to evaporate in room temperature with a constant flow of N_2. When the tubes were dry, they were diluted with 250µl MeOH and left for 5 minutes in room temperature followed by vortexing for 10 seconds and centrifuged 2 minutes at 16000g at 4˚C. The supernatants were transferred into vials for analysis

on LC/MS. Standards were prepared by using different ratios of MDA, HHE (1mg in 100µl MeOH) and HNE (1mg in 100µl EtOH). The MDA was prepared with 1mM 1,1,3,3-tetraethoxypropane (TEP) hydrolysed in 1% H_2SO_4 for 120min at 25˚C [49].

The analyses were performed on an *Agilent 1260 HPLC system* connected to a binary pump, an autosampler, a column oven with a *Phenomenex Luna* (250mm×4.6mm×3µm) C18 column with a gradient at 50˚C and a UV-detector coupled to an *Agilent 6120 quadrupole* in the APCI negative mode. The sample injection volume was 15µl and the flowrate of the mobile phase was 700µl/min. The two mobile phases consisted of 20mM acetic acid (HAc) in water (A) and MeOH (B). The program started out with 30% A and 70% B for 2 minutes. For the following 8 minutes, the ratio changed linearly to 5% A and 95% B. This setting was kept for 10 minutes whereafter another linear increase of mobile phase B to 98% was performed during 5 minutes. This setting was kept stable during 2 minutes before mobile phase B decreased to 70% during 1 minute. This setting was then kept until the end of the program after 40 minutes. N_2 was used as a nebulizer gas at 40psig, the drying and vaporization temperature was at 350˚C and 450˚C respectively. The data was analyzed in the in selected ion monitoring (SIM) mode, using the *Agilent ChemStation* software. Molecules of the mass to charge ratio (m/z) 234, 293 and 335 were collected as ions corresponding to MDA, HHE and HNE when derivatized with DNPH.

3.3.3.4 Sensory analysis of rancid odor

A panel of two trained panelists took part in a blind test where they estimated the rancid odor of the different oils by drawing an X on a 10cm line corresponding to a rancidity intensity of 0-100. The sensory analysis was done after 0, 3, 7, 10, 15, 21 and 28 days.

3.3.4 Lipid oxidation during in vitro digestion

The *in vitro* digestion was performed by following a standardized *in vitro* digestion method developed by *Minekus et al.* (2014) hereafter referred to as the Infogest-protocol [50]. Reagent grade salts together with enzymes and extracts originating from fungi and porcine were used to create simulated digestive fluids. To be able to prepare the simulated fluids, enzymatic activity had to be measured through a number of assays described in **Appendix D**. Activities of enzymes can be seen in **Table 6**. Simulated salivary, gastric and intestinal fluids (SSF, SGF, SIF) were prepared as seen in **Table 7**. The salt solutions were prepared beforehand and stored in the freezer (-20˚C) in volumes of 10ml in 13ml centrifuge tubes. On the day of digestion, 2.5ml of the enzymes, $CaCl_2$ and bile salts were mixed with the 10ml to give the final concentrations as seen in **Table 7**.

Table 6. *Measured activities of enzymes used during digestion. See* ***Appendix D*** *for definitions of U and TBU*

Enzyme	Activity
α-amylase from porcine pancreas (A3176, *Sigma-Aldrich*)	1.60 ± 0.35 U/mg
Lipase from Rhizopus oryzae (80612, *Sigma-Aldrich*)	7.24 ± 0.61 TBU/mg
Pepsin from porcine gastric mucosa (P7000, *Sigma-Aldrich*)	705.99 ± 19.18 U/mg
Lipase in *Pancreatin from porcine pancreas* (P1750, *Sigma-Aldrich*)	34.24 ± 3.44 TBU/mg
Trypsin in *Pancreatin from porcine pancreas* (P1750, *Sigma-Aldrich*)	2.68 ± 0.17 U/mg

Table 7. *Concentration of salts and enzymes in SSF, SGF and SIF. The numbers given within brackets are the concentrations/activities in the reaction mixture when taking the step-by-step addition and dilution factor into account*

Salts	**SSF [mM]**		**SGF [mM]**		**SIF [mM]**	
KCl	15.1	(7.550)	6.9	(7.230)	6.8	(7.013)
KH_2PO_4	3.7	(1.850)	0.9	(1.375)	0.8	(1.088)
$NaHCO_3$	13.6	(6.800)	25	(15.90)	85	(50.45)
NaCl			47.2	(23.60)	38.4	(31.00)
$MgCl_2(H_2O)_6$	0.15	(0.075)	0.12	(0.100)	0.33	(0.214)
NH_4CO_3	0.12	(0.060)	1	(0.530)		(0.265)
Enzymes	**SSF [U/ml]**		**SGF [U/ml]**		**SIF [U/ml]**	
α-amylase from porcine pancreas (A3176, *Sigma-Aldrich*)	150	(75.00)				
Lipase from Rhizopus oryzae (80612, *Sigma-Aldrich*)			16	(8.000)		
Pepsin from porcine gastric mucosa (P7000, *Sigma-Aldrich*)			4000	(2000)		
Lipase in *Pancreatin from porcine pancreas* (P1750, *Sigma-Aldrich*)					2555	(1278)
Trypsin in *Pancreatin from porcine pancreas* (P1750, *Sigma-Aldrich*)					200	(100.0)
Further additions	**SSF [mM]**		**SGF [mM]**		**SIF [mM]**	
$CaCl_2(H_2O)_2$	1.5	(0.750)	0.15	(0.045)	0.6	(0.525)
Bile salts					20	(10.00)

For the SSF, the concentration of α-amylase together with the associated concentration of $CaCl_2$ was decided according to the Infogest-protocol. For the SGF, the concentration of lipase from *Rhizopus oryzae* was decided to be 8U/ml [31, 51]. The concentration of pepsin together with the associated concentration of $CaCl_2$ was decided according to the Infogest-protocol. It was assumed that the calcium present in the SGF was enough to maintain the lipase active. For the SIF, the concentration of pancreatin was based on trypsin activity as in the Infogest-protocol. The lipase activity then naturally follows

to the activity shown in **Table 7**. According to data acquired from *Zangenberg et al.* (2001), *Porcine bile extract* (B8631, *Sigma-Aldrich*) contained 49% (w/w) bile salts [52]. The net molecular weight of bile salts in porcine bile extract is 483.13 g/mol [53]. To get a concentration of 10mM in the final mixture according to the Infogest-protocol, 246.5mg was included in the SIF, giving a bile extract concentration of 2.0% (w/v). A similar concentration was used by *Larsson et al.* (2012) when 2.5% of the SIF was bile extract [54].

To monitor the production of selected reactive aldehydes during the course of *in vitro* digestion, 4 withdrawal points were chosen. The first withdrawal was after weighing the oils and the second to fourth withdrawal at the beginning (after the addition of SIF), middle and end of the intestinal stage. The digestion was done in duplicate on different days and began with adding 50mg of pure and fortified fish oils to 200µl mQ-H_2O in separate 5ml Eppendorf tubes. Blanks for each intestinal withdrawal point was also prepared, as *Porcine bile extract* contains some fats which are prone to undergo lipid peroxidation. These blanks contained 250µl mQ-H_2O. To initiate the mouth phase 250µl SSF was added, raising the pH to 7. The tube was then vortexed for 2 seconds. After 2 minutes the gastric phase was initiated by adding 500µl SGF and adjusting the pH to 6.5 with the addition of 10µl 1M HCl. The tubes were flushed with N_2 for 4 seconds, before closing the lid and vortexing for 2 seconds. From this point forward the tubes were always flushed with N_2 for 4 seconds and vortexed for 2 seconds when opened. The tubes were then incubated in darkness in 37˚C on an *ELMI S3.01.016* orbital shaker (50rpm) for 60 minutes before the pH was decreased to 2.8 by adding 12µl 1M HCl. The decrease in pH in the gastric phase was done according to the study performed by *Sams et al.* (2016), showing that the pH in our gut increase to 5-7 after the intake of food, to then dynamically return to pH 1-1.5 after 3 hours [55]. After another 60 minutes of incubation, the intestinal phase was initiated by adding 1000µl SIF, raising the pH to 7. The tubes were then put back in the incubator on the orbital shaker (200rpm) for 90 minutes before ending the *in vitro* digestion. The samples were stored in -80˚C until analyzed.

3.3.4.1 REACTIVE ALDEHYDES

Approximately 80mg of the pure and fortified fish oil from the first withdrawal point was mixed with 420µl mQ-H_2O to work as zero samples. This was done in order to get an understanding of the oxidation-status of the different fish oils before they entered the *in vitro* digestive system. The other samples, including the blanks, consisted of a 500µl digestive fluid from the beginning, middle and end of the intestinal stage. The aldehydes were analyzed as described in part 3.3.3.3.

4 RESULTS

4.1 CRUDE COMPOSITION OF SEAWEED BIOMASS

4.1.1 DRY WEIGHT

Porphyra had the dry weight of 15.3% ± 0.4, and *Ulva* had the dry weight of 18.6% ± 0.3.

4.1.2 TOTAL LIPID CONTENT

Porphyra had a fat content of 2.0% ± 0.2, and *Ulva* had a fat content of 2.1% ± 0.4 dry weight basis.

4.1.3 FATTY ACID COMPOSITION

The fatty acid composition for *Porphyra* and *Ulva* can be seen in **Table 8.** *Porphyra* contained no short chained (SC) n-3 PUFAs, but 2.9% SC n-6 PUFAs. However, it contained 42.8% LC n-3 PUFAs (42.1% EPA), and 19.2% LC n-6 PUFAs. *Ulva* on the other hand contained 38.9% SC n-3 PUFAs (20.8% ALA), and 12.8% SC n-6 PUFAs. When it comes to the longer more beneficial PUFAs, *Ulva* only contained 4.0% LC n-3 PUFAs and 2.1% LC n-6 PUFAs. **Table 8** shows that the two species have a very different composition of fatty acids, where the *Ulva* have more SC fatty acids and the *Porphyra* more LC fatty acids in contrast to one another. Based on the seaweed dry weight, the total fatty acids contributed with 2.23% ± 0.009 of the *Porphyra* and 2.14% ± 0.003 of the *Ulva*.

Table 8. *Relative fatty acid composition of total fatty acids (% of total fatty acids) from Porphyra and Ulva ± standard deviation (n=2). N/A = not available*

Fatty acid	*Porphyra*	*Ulva*
C14:0	0.31% ± 0.02%	0.43% ± 0.02%
C15:0	0.18% ± 0,00%	0.08% ± 0,00%
C16:0	25.21% ± 0.47%	22.32% ± 0.82%
C16:1	0.93% ± 0.04%	3.12% ± 0,00%
C16:2	0,00% ± 0,00%	1.74% ± 0.01%
C16:4 n3	N / A	9.37% ± 0.07%
C17:1	N / A	3.68% ± 0.05%
C18:0	0.59% ± 0.01%	0.15% ± 0,00%
C18:1	5.84% ± 0.13%	9.83% ± 0.04%
C18:2 n6 - LA	2.48% ± 0.05%	10.86% ± 0.01%
C18:3 n6	0.44% ± 0.01%	1.96% ± 0.05%
C18:3 n3 - ALA	N / A	20.80% ± 0.08%
C18:4 n3	N / A	8.68% ± 0.03%
C20:1 n9	1.89% ± 0.02%	0.06% ± 0.01%
C20:2 n6	0.74% ± 0.04%	0.12% ± 0.02%
C20:3 n6	6.42% ± 0.02%	0.42% ± 0.02%
C20:4 n6 - AA	11.99% ± 0.22%	0.98% ± 0.05%
C20:4 n3	0.71% ± 0.05%	0.35% ± 0.03%
C20:5 n3 - EPA	42.07% ± 0.85%	0.99% ± 0.05%
C22:0	N / A	0.58% ± 0.02%
C22:1 n9	0.21% ± 0,00%	0.32% ± 0.05%
C22:4 n6	N / A	0.53% ± 0.12%
C22:5 n3	N / A	2.62% ± 0.32%
C22:6 n3 - DHA	N / A	N / A

4.1.4 *Pigments and phenolic compounds*

See **Figure 9** for a picture of *Porphyra* and *Ulva* extracted in 80% acetone. Notice that even the red seaweed yields a green acetone extract. The result of chlorophyll, carotenoids and total phenolic compounds can be seen **Figure 10**. The *Ulva* contained more of each lipophilic compound compared to *Porphyra*.

Figure 9. *Picture of Porphyra (left) and Ulva (right) extracted in 80% acetone*

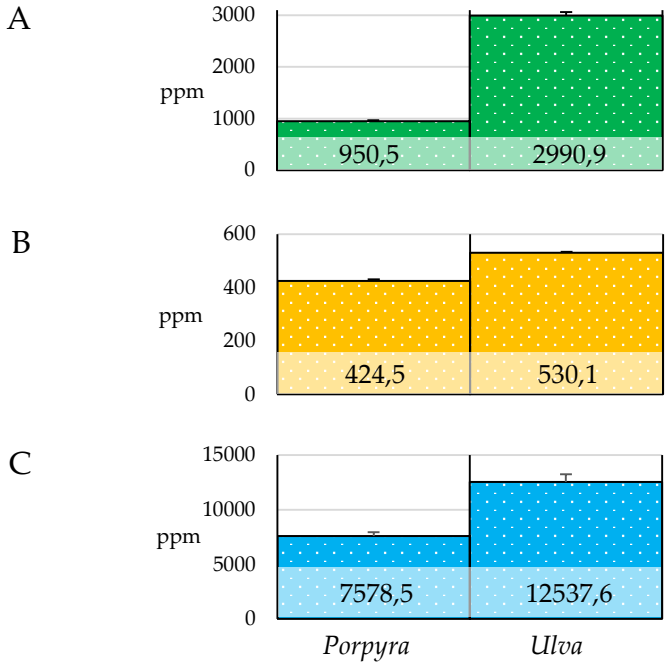

Figure 10. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted from dry Porphyra and Ulva ± standard deviation (n=3)*

4.2 Extraction of seaweed into sunflower oil

In this section, the results from the different physical extraction methods of seaweed in sunflower oil are evaluated. The extractions are graded based on their ability to increase the concentration of chlorophyll, carotenoids and phenolic compounds, and change the color of the oil. Sunflower oil was used in this aspect due to its low content of LC n-3 PUFA, thus facilitating the chances of finding an LC n-3 PUFA enrichment.

4.2.1 *Optimization of extraction methods*

Diagrams showing extracted lipophilic compounds and total change in color for all extraction methods can be seen in **Appendix B** together with the statistical data (F-values) for the methods where factorial design was used. The temperature profile for all extraction methods is shown in **Figure 11**.

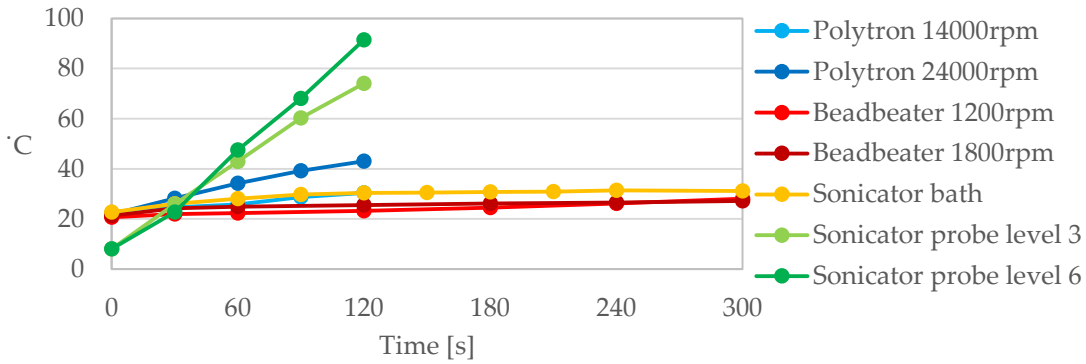

Figure 11. *Temperature profile for all extraction methods*

4.2.1.1 POLYTRON

As can be seen in **Figure 11** the temperature of the sunflower oil reached 30˚C and 43˚C after 120 seconds at 14000rpm and 24000rpm respectively. Extracting seaweed with the polytron at these settings for 60 and 120 seconds lead to the highest concentrations of chlorophyll and carotenoids being extracted in the oil fortified by *Ulva*. The oil were also darker, more green and yellow, compared to the oil fortified by *Porphyra*. Extraction with wet seaweed lead to more chlorophyll A being extracted. Running the extraction two minutes instead of one increased the concentration of chlorophyll and carotenoids, and also lead to a darker, more green and yellow oil compared to running the extraction one minute. The higher frequency level increased extraction of chlorophyll and carotenoids, but decreased the concentration of total phenolic compounds in the sunflower oil. It also lead to the fortified oils being darker, more green and yellow. The significant (α=0.05) increase in pigments and total difference in color makes the polytron setting at 24000 for 2 minutes the most successful.

4.2.1.2 BEADBEATER

The temperature profile for the beadbeater run at 1200 and 1800rpm is shown in **Figure 11**. The final temperature in the sunflower oil after 5 minutes did not exceed 30 ˚C at neither frequency. The only difference found was that the amount of chlorophyll A increased when extracting *Ulva* compared to *Porphyra*. The state, time and frequency did not have any impact on the extraction. However, when analyzing the statistics for the second run by itself, the concentration of total chlorophyll was higher when extracting *Ulva* compared to when extracting *Porphyra*. Longer extraction time lead to more carotenoids being extracted. Higher frequency lead to more carotenoids and less phenolic compounds being extracted. The concentration of carotenoids was still less compared to what was achieved with the polytron. The high concentration of chlorophyll compared to carotenoids made this method less interesting than the polytron. When looking at the concentration of total phenolic compounds, it was in general higher compared to what was achieved with the polytron. When it comes to the change in total color, sunflower oil extracted with *Ulva* had a darker, more green and yellow color. Extracting wet seaweed lead to a darker oil, compared to when extracting dry seaweed. A prolonged extraction time lead to an increased green and

yellow color of the oil. Higher power level lead to a darker, more green and yellow oil. The final choice of method was to run the beadbeater at its highest frequency (1800rpm) for 5 minutes. The choice of wet or dry biomass will be further discussed at a later stage.

4.2.1.3 SONICATOR BATH

The temperature profile for the sonicator bath shown in **Figure 11** went stagnant at 31˚C, fluctuating a bit up and down until the end of the experiment at 300 seconds. The total color of the fortified oil could not be recorded for the dry samples, as the dry seaweed soaked up the oil, leaving too little behind for measuring both the absorbance and the color. The concentration of total phenolic compounds between species and states were significantly (α=0.05) different. *Porphyra* in the wet state gave the highest concentration of phenolic compounds. The oil fortified with *Ulva* gave once again rise to the greenest color. Due to optimization limitations, since the equipment only had one setting, this experiment was only conducted once. A longer extraction time should lead to further extraction; hence 5 minutes was chosen as the optimal setting. Altogether, the sonicator bath was considered to contribute with little added value, compared to what could be extracted with simply just letting the seaweed lay in the sunflower oil for 3 hours. This hypothesis was tested in part 4.2.2.

4.2.1.4 SONICATOR PROBE

The temperature profile for the sunflower oil during evaluation of the sonicator probe is shown in **Figure 11**. After 70 seconds of sonicating, the temperature was just below 50˚C for both output levels, which was a big increase in temperature compared to previous methods. The temperature reached 100˚C after 203 and 140 seconds for the medium and high output level, respectively.

Due to measurement of phenolic compounds giving vague results with high variation, it was excluded in the optimization of the sonicator probe. Instead the optimization was performed based on extraction of pigments and total change in color. The result from the first factorial design showed that the combined effect of duty cycle and output level was significant (α=0.05) when looking at the total chlorophyll concentration. Meaning that extractions where both duty cycle and output level were either high or low, were the most successful ones. The same interaction effect also yielded a more green oil. It is probable that this could be explained by the different extraction times needed for the oil to reach 30˚C. Reaching this temperature took 155 seconds when both settings were low, and 106 seconds when both settings were high. In the other points, reaching 30˚C took between 27 and 38 seconds. The oils fortified on output level 6 became darker, more green and yellow oils compared to when output level 2 was used. For all color measurement, the pure quadratic effect of the center point was significant (α=0.05), indicating that an optimum setting might exist within the experimental design.

For the second experimental setup, output level 5 yielded a greener and more yellow fortified oil compared to both output level 4 and 6. This lead to output level 5 being the most successful setting. However, it was still unclear which duty cycle should be selected. A third test was conducted where the whole span of duty cycles (40-80%) with a 10% interval on output level 5 were tested. Statistical analysis could not describe any significant (α=0.05) change in pigment concentration, nor color change related to duty cycle. When the duty cycle was 60%, the highest level of extracted carotenoids was recorded. The same was observed when it came to the total change in color, where a duty cycle at 60% yielded a darker more green and yellow fortified oil. This is the duty cycle setting which was chosen as the optimal setting, to be used in the proceeding experiments. A fourth test with the sonicator probe was conducted, to check whether dry *Porphyra* had the same optimum for duty cycle and output level. Statistical analysis could only show that output level 5 yielded the most yellow oil compared to output level 4 and 6. As this was the only thing that could be concluded from the experiment with dry *Porphyra*, the optimal settings remained on output level 5 and duty cycle 60% for the future experiments.

4.2.2 Comparison of the extraction methods

A picture of the fortified oils can be seen in **Figure 12**. The extraction of pigments and phenolic compounds from *Porphyra* can be seen in **Figure 13**, and the total change in color can be seen in **Figure 14**. The same data for *Ulva* is represented in **Figure 15** and **16** respectively. The samples that were stored in room temperature for 3 hours, and were subjected to vortex 4 times, had higher levels of carotenoids extracted than both the beadbeater and sonicator bath. This fact made both these methods less interesting, as they do not provide any additional value. The polytron was most successful for extraction of pigments, and the sonicator probe most successful for extraction of phenolic compounds. The sonicator probe also showed low concentration of extracted chlorophyll compared to the polytron. These two extraction methods were considered the two most promising. Another experiment was conducted comparing these two methods, where the extraction temperature was taken into account.

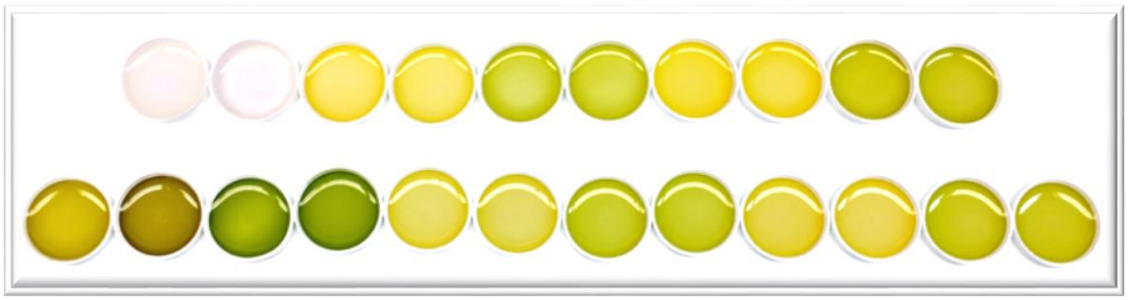

Figure 12. *Picture of the oils in duplicates when measuring color. On the first row from left to right: pure sunflower oil, Porphyra and Ulva with beadbeater, Porphyra and Ulva vortexed in room temperature. On the second row from left to right: Porphyra and Ulva with polytron, Porphyra and Ulva in sonicator bath, Porphyra and Ulva with sonicator probe.*

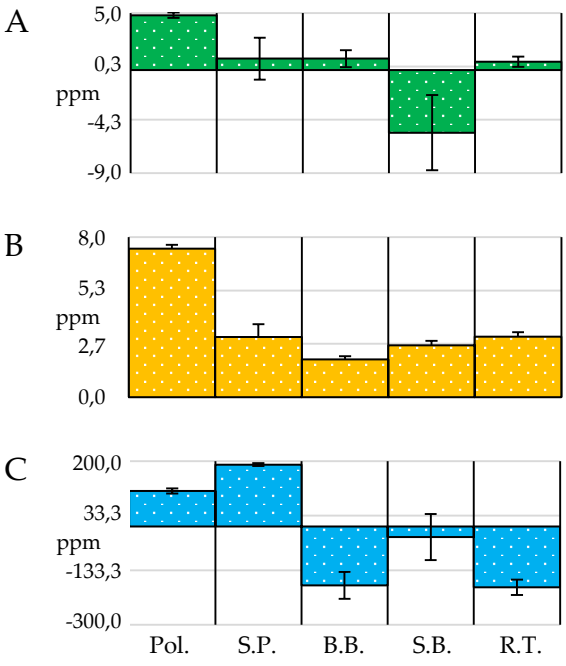

Figure 13. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted from Porphyra into sunflower oil ± standard deviation (n=2)*

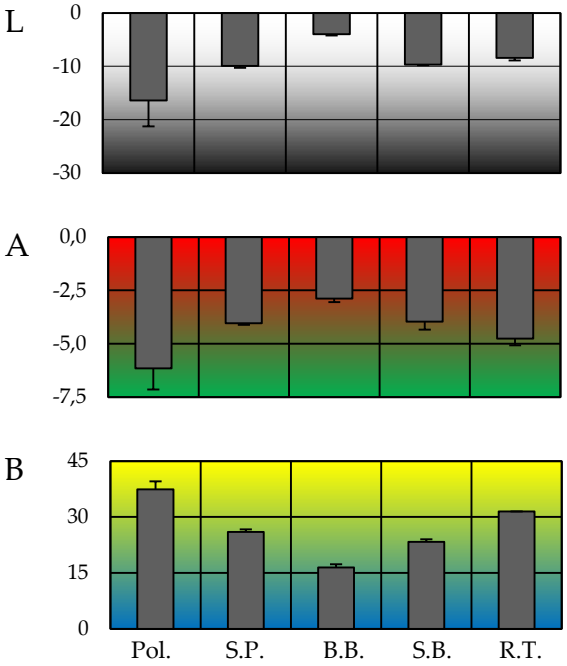

Figure 14. *L-, a- and b-value for sunflower oil after extraction of Porphyra using different methods ± standard deviation (n=2)*

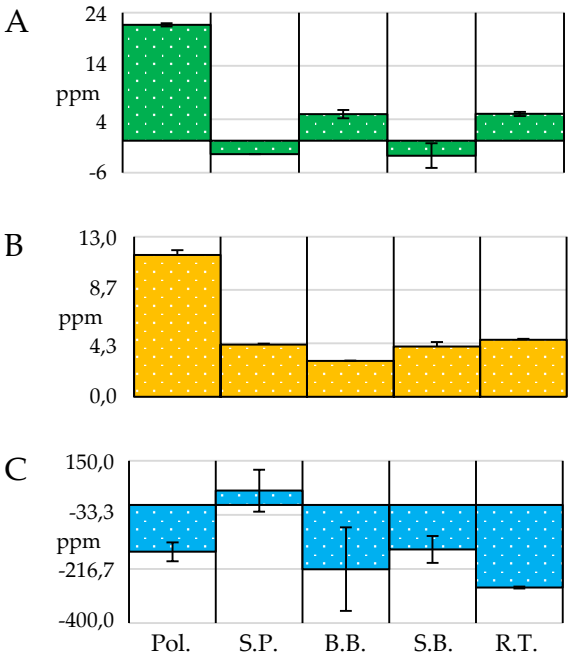

Figure 15. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted from Ulva into sunflower oil ± standard deviation (n=2)*

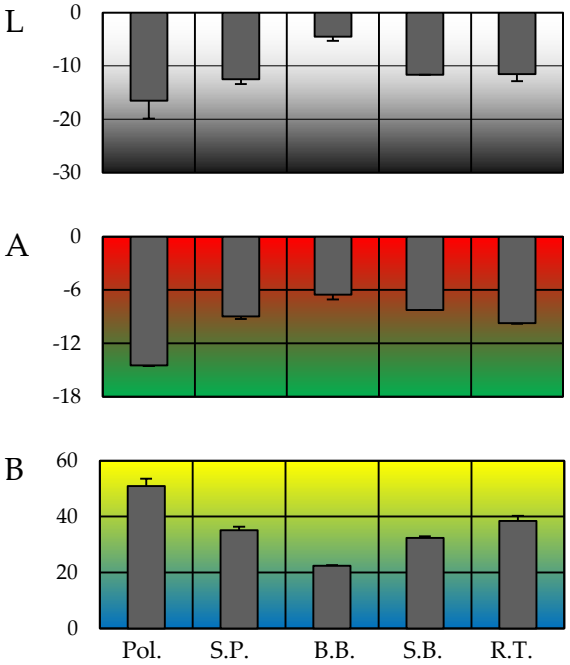

Figure 16. *L-, a- and b-value for sunflower oil after extraction of Ulva using different methods ± standard deviation (n=2)*

4.2.3 *Effect of heat on extraction*

The extraction of pigments and phenolic compounds from *Porphyra* can be seen in **Figure 17**, and the total change in color can be seen in **Figure 18**. To reach 30˚C in the oil, the polytron had to be run for 1 minute, meanwhile it took 5 minutes to reach 50˚C. The increased duration could explain the high level of carotenoids detected after the 5 minutes extraction, which can be seen in **Figure 17**. For the oils heated in the oven, it took 10 minutes for the oils to reach 50˚C. The significantly (α=0.05) higher carotenoid level found in the sample extracted with the polytron to 50˚C proves that the polytron adds an increased value as an extraction method. The sonicator probe have previously shown good result in extraction of phenolic compounds. However, in this test, due to large deviation, no difference was found between the methods ability to extract phenolic compounds. For the sonicator probe; this together with the low carotenoid extraction made the sonicator probe loose value. The color of the oils extracted with the polytron to 50˚C were the darkest, and greener than the oils from the sonicator probe. The results acquired from analysis of pigments and total change in color indicates that using the polytron until the oil temperature reached 50˚C, was the most efficient method in extracting lipophilic compounds. It was further tested if this method was able to fortify sunflower oil with additional fatty acids from the seaweed.

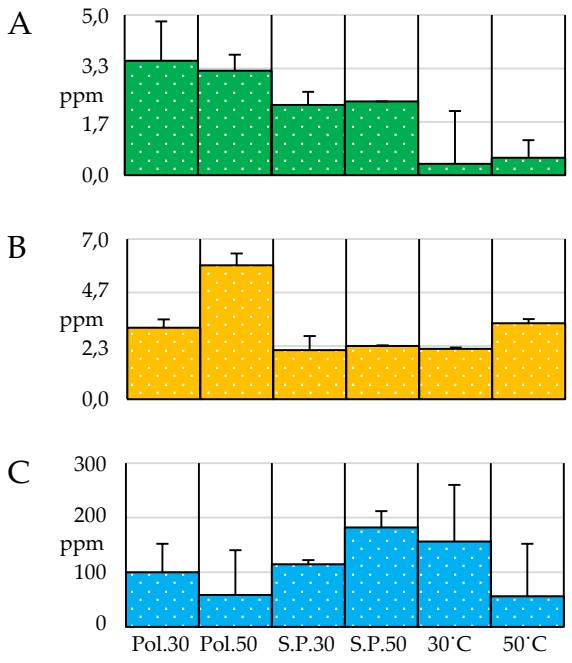

Figure 17. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted from Porphyra into sunflower oils using different methods in which temperatures reached 30 and 50˚C ± standard deviation (n=2)*

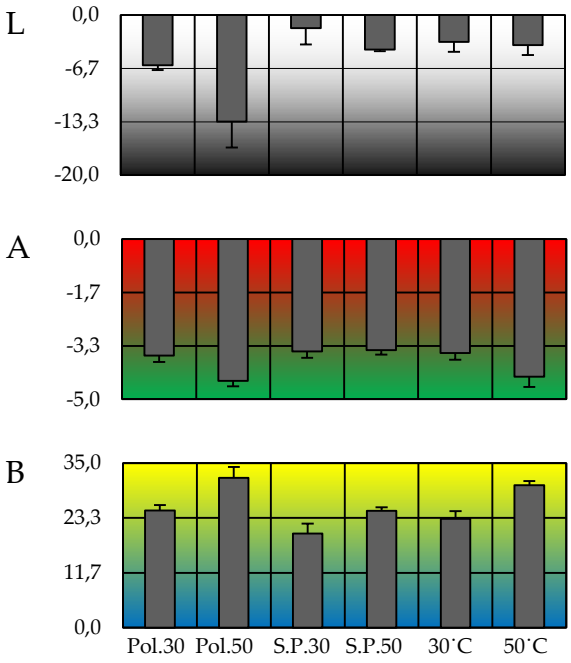

Figure 18. *L-, a- and b-value for sunflower oil after extraction of Porphyra using different methods in which temperatures reached 30 and 50˚C ± standard deviation (n=2)*

4.2.4 *Analysis of the most promising method*

The fatty acid composition of pure sunflower oil and fortified sunflower oil, when using the polytron to 50˚C to extract *Porphyra* and *Ulva* can be seen in **Table 9**. The fortified sunflower oils contained significantly (α=0.05) more C17:1 n7 compared to the pure sunflower oil, as the concentration increased with 9%. The sunflower oil fortified with *Ulva* contained significantly (α=0.05) more ALA (C18:3 n3) than the other oils, as the concentration increased with 11%, see **Figure 19**.

***Table 9**. Fatty acid composition of pure and fortified sunflower oil (%w/w, on an oil basis) ± standard deviation (n=2)*

Fatty acid	Pure sunflower oil	Fortified with *Porphyra*	Fortified with *Ulva*
C14:0	0.05% ± 0,00%	0.05% ± 0,00%	0.05% ± 0,00%
C15:0	0.01% ± 0,00%	0.01% ± 0,00%	0.01% ± 0,00%
C16:0	5.62% ± 0.16%	5.70% ± 0.06%	5.70% ± 0.01%
C16:1	0.09% ± 0,00%	0.09% ± 0,00%	0.08% ± 0,00%
C17:1 n7	**0.02% ± 0,00%**	**0.03% ± 0,00%**	**0.03% ± 0,00%**
C18:0	3.17% ± 0.09%	3.22% ± 0.06%	3.18% ± 0,00%
C18:1 n9	24.57% ± 0.75%	24.66% ± 0.72%	25.03% ± 0.31%
C18:1 n7	0.72% ± 0.02%	0.70% ± 0.03%	0.72% ± 0.04%
C18:2 n6 - LA	53.02% ± 1.69%	52.88% ± 2.21%	54.01% ± 0.55%
C18:3 n3 - ALA	**0.04% ± 0,00%**	**0.04% ± 0,00%**	**0.05% ± 0,00%**
C20:0	0.19% ± 0,00%	0.20% ± 0,00%	0.19% ± 0,00%
C20:1 n9	0.10% ± 0.01%	0.10% ± 0,00%	0.10% ± 0,00%
C22:0	0.68% ± 0.01%	0.70% ± 0.02%	0.67% ± 0,00%
C24:0	0.18% ± 0,00%	0.18% ± 0.01%	0.18% ± 0.01%
Total	88.47% ± 2.75%	88.57% ± 3.03%	90,00% ± 0.84%

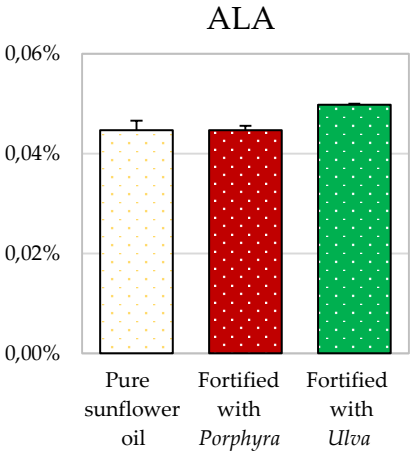

***Figure 19.** Concentration of ALA in the pure sunflower oil and sunflower oil fortified with Porphyra and Ulva ± standard deviation (n=2)*

4.3 Extraction of seaweed into fish oil

4.3.1 *Effect of heat on lipid peroxidation in fish oil*

To elucidate if the heat generated by the extraction methods had an effect on oxidation of the oils, PV was studied as a function of temperature. Heating fish oil to 50˚C did significantly (α=0.05) increase the concentration of peroxides by approximately 0.25µmol/g fish oil, see **Figure 20**. According to the research performed by *Fakourelis et al.* (1987), a 5ppm concentration of carotenoids in olive oil contained 0.32µmol/g less peroxides after only 12 hours of light storage at room temperature when compared to an olive oil without carotenoids [35]. This fact supported that the extraction method could be used on seaweed in fish oil, as the extracted amounts of carotenoids had been previously measured to be ~6ppm.

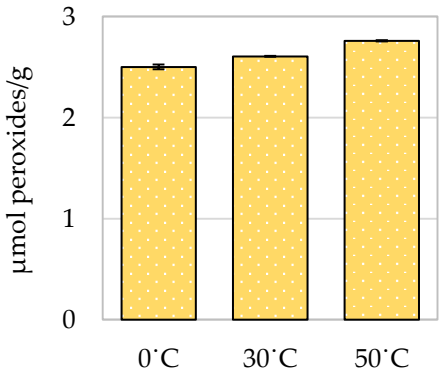

***Figure 20.** Concentration of peroxides in the fish oil samples ± standard deviation (n=2) after heating to 30˚C and 50˚C*

4.3.2 FORTIFICATION OF FISH OIL

4.3.2.1 PIGMENTS AND PHENOLIC COMPOUNDS

The pigments and phenolic compounds detected in the pure and fortified fish oil are shown in **Figure 21**. The concentration of chlorophyll and carotenoids were significantly (α=0.05) higher in the fish oils fortified with *Porphyra* and *Ulva*, compared to pure fish oil. Furthermore, the concentrations were significantly (α=0.05) higher in the fish oil fortified with *Ulva* compared to the fish oil fortified with *Porphyra*.

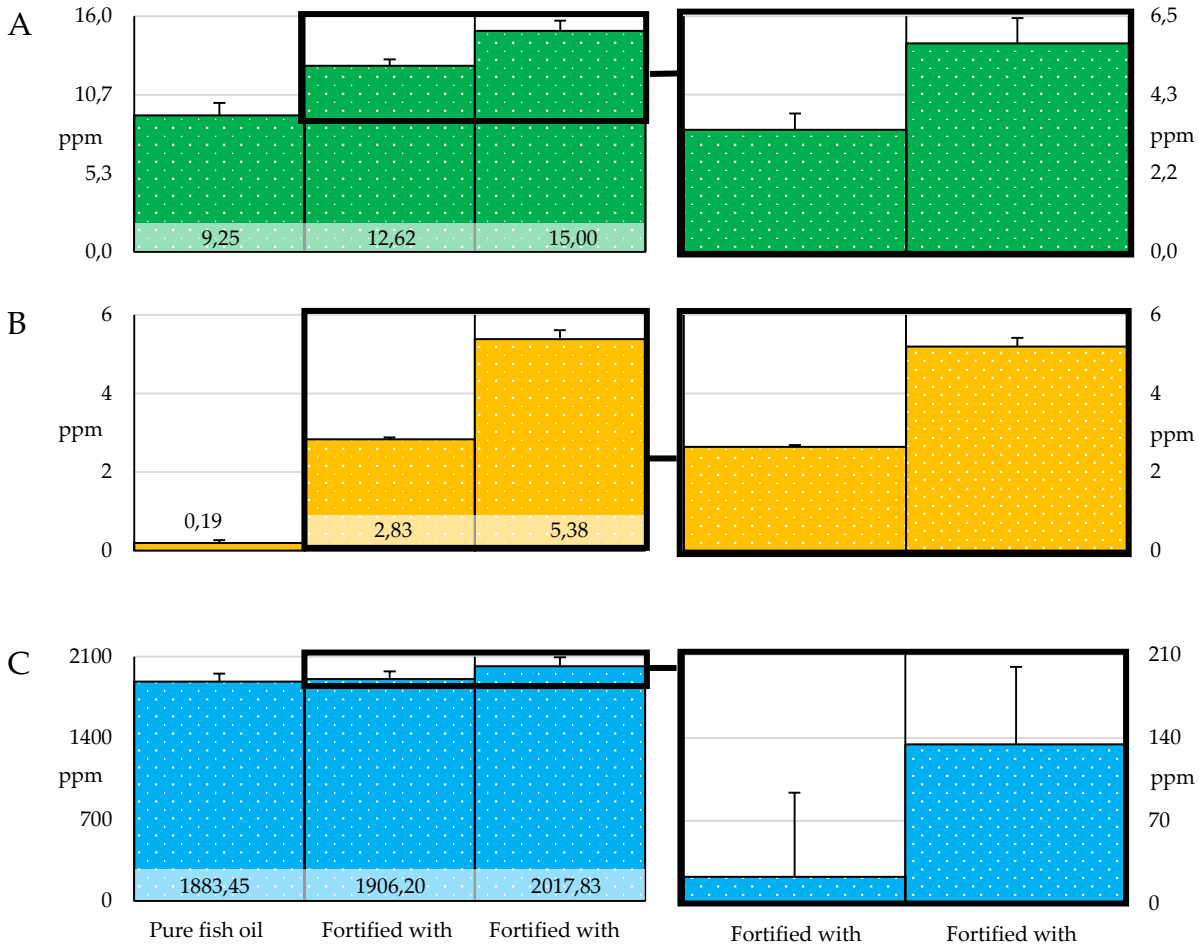

Figure 21. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds in pure and fortified fish oil when using the polytron to 50˚C ± standard deviation (n=3). The right panel shows the increase of lipophilic compounds compared to pure fish oil*

4.3.3 STORAGE STUDY OF PURE AND FORTIFIED FISH OIL

The appearance of the oils at the day of fortification is shown in **Figure 22 (A)**. After only 3 days of exposure to daylight at room temperature, a pellet had formed on the bottom of the tubes with the fortified oils, leaving a clearer colorless oil as a supernatant, see **Figure 22 (B)**. The most drastic change after 3 days of storage appeared for the fish oil fortified with *Ulva* which lost its green appearance. The following parts will treat results from storage of the pure and fortified fish oils exposed to daylight at room temperature. For weekly pictures of the oils stored in daylight and in darkness in room temperature, and in darkness in the fridge, see **Appendix C**.

A B

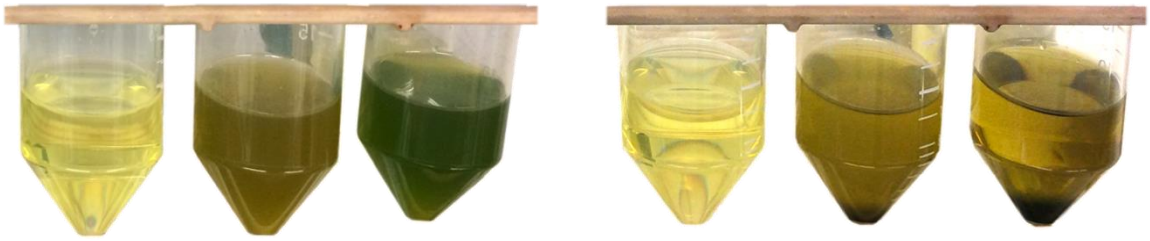

Figure 22. *Picture of from left to right: pure fish oil and fish oil fortified with Porphyra and Ulva at (A) day of fortification and (B) after 3 days of daylight storage at room temperature*

4.3.3.1 CHANGES IN FATTY ACID COMPOSITION

The fatty acid composition of the oils before and after 28 days of storage can be seen in **Table 10** and **11** respectively. In **Table 10** the fortified oils contain the same or less concentration of each fatty acid, compared to the pure fish oil. In **Table 11**, the amount of ALA, EPA and DHA was significantly (α=0.05) higher in the fish oil fortified with *Ulva* compared to the pure fish oil. The decrease in ALA, EPA and DHA over 28 days' time can be seen in **Figure 23**. The top standard deviation explains the different amount of each fatty acid initially (**Table 10**). The standard deviation at the bottom describes the changes in the oils after 28 days storage (**Table 11**). The degradation of EPA and DHA was 28% less in the fish oil fortiafied with *Porphyra* and 22% and 37% less in the fish oil fortified with *Ulva* compared to the pure fish oil.

Table 10. *Fatty acid composition of pure and fortified fish oil (%w/w, on an oil basis) after 0 days of daylight storage at room temperature ± standard deviation (n=3)*

Fatty acid	Pure fish oil	Fortified with *Porphyra*	Fortified with *Ulva*
<C18:0	19.83% ± 0.1%	19.52% ± 0.09%	19.75% ± 0.02%
C18:0	1.69% ± 0.02%	1.67% ± 0.02%	1.68% ± 0,00%
C18:1	18.56% ± 0.14%	18.15% ± 0.17%	18.66% ± 0.17%
C18:2 n6 - LA	1.53% ± 0.01%	1.53% ± 0.02%	1.54% ± 0.02%
C18:3 n6	0.11% ± 0.01%	0.12% ± 0,00%	0.12% ± 0,00%
C18:3 n3 - ALA	0.66% ± 0,00%	0.66% ± 0,00%	0.66% ± 0,00%
C18:4 n3	2.23% ± 0.03%	2.2% ± 0.03%	2.2% ± 0.01%
C20:1	10.02% ± 0.07%	9.91% ± 0.08%	10,00% ± 0.05%
C20:2 n6	0.26% ± 0,00%	0.25% ± 0.01%	0.25% ± 0,00%
C20:3 n3	0.13% ± 0,00%	0.13% ± 0,00%	0.13% ± 0,00%
C20:4 n6 - AA	0.37% ± 0,00%	0.37% ± 0.01%	0.35% ± 0,00%
C20:4 n3	0.79% ± 0.01%	0.79% ± 0.02%	0.74% ± 0.01%
C20:5 n3 - EPA	7.9% ± 0.02%	7.85% ± 0.04%	7.88% ± 0.03%
C21:5 n3	0.4% ± 0,00%	0.4% ± 0.01%	0.39% ± 0,00%
C22:1	8.79% ± 0.3%	8.55% ± 0.06%	8.59% ± 0.01%
C22:5 n6	0.11% ± 0,00%	0.11% ± 0.01%	0.1% ± 0,00%
C22:5 n3	1.12% ± 0.01%	1.12% ± 0.03%	1.11% ± 0.01%
C22:6 n3 - DHA	10.92% ± 0.03%	10.85% ± 0.07%	10.88% ± 0.06%
C24:1 n9	0.47% ± 0.02%	0.46% ± 0.01%	0.46% ± 0.01%
Total	85.9% ± 0.56%	84.66% ± 0.29%	85.5% ± 0.19%

Table 11. *Fatty acid composition of pure and fortified fish oil (%w/w, on an oil basis) after 28 days of daylight storage at room temperature ± standard deviation (n=3)*

Fatty acid	Pure fish oil	Fortified with *Porphyra*	Fortified with *Ulva*
<C18:0	19.16% ± 0.23%	19.39% ± 0.25%	19.27% ± 0.15%
C18:0	1.63% ± 0.02%	1.66% ± 0.05%	1.63% ± 0.02%
C18:1	18.29% ± 0.29%	18.2% ± 0.24%	18.32% ± 0.07%
C18:2 n6 - LA	1.42% ± 0.01%	1.47% ± 0.07%	1.43% ± 0.01%
C18:3 n6	0.1% ± 0,00%	0.1% ± 0.01%	0.09% ± 0,00%
C18:3 n3 - ALA	0.6% ± 0.01%	0.61% ± 0.02%	0.61% ± 0,00%
C18:4 n3	1.98% ± 0.02%	2.01% ± 0.02%	2.01% ± 0.02%
C20:1	9.79% ± 0.16%	9.72% ± 0.07%	9.76% ± 0.01%
C20:2 n6	0.22% ± 0,00%	0.23% ± 0.01%	0.22% ± 0.01%
C20:3 n3	0.12% ± 0,00%	0.12% ± 0.01%	0.11% ± 0,00%
C20:4 n6 - AA	0.32% ± 0,00%	0.34% ± 0.03%	0.33% ± 0,00%
C20:4 n3	0.67% ± 0.01%	0.68% ± 0.03%	0.66% ± 0,00%
C20:5 n3 - EPA	7.18% ± 0.09%	7.34% ± 0.14%	7.31% ± 0.04%
C21:5 n3	0.35% ± 0.01%	0.36% ± 0.01%	0.36% ± 0,00%
C22:1	8.33% ± 0.08%	8.39% ± 0.08%	8.4% ± 0.04%
C22:5 n6	0.1% ± 0.01%	0.1% ± 0,00%	0.09% ± 0.01%
C22:5 n3	1,00% ± 0.03%	1.01% ± 0.03%	1.01% ± 0,00%
C22:6 n3 - DHA	9.95% ± 0.15%	10.16% ± 0.11%	10.27% ± 0.12%
C24:1 n9	0.46% ± 0.02%	0.49% ± 0.1%	0.47% ± 0.02%
Total	81.65% ± 1.01%	82.38% ± 0.78%	82.37% ± 0.36%

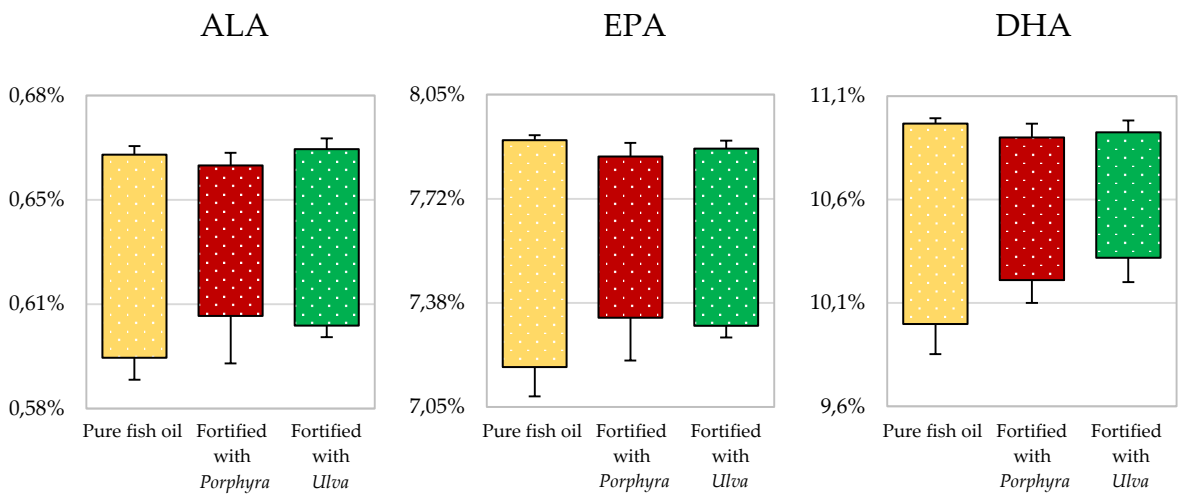

Figure 23. *Decrease of ALA, EPA and DHA fatty acids in the pure fish oil and fish oils fortified with Porhyra and Ulva after 28 days of daylight storage at room temperature ± standard deviation (n=3)*

4.3.3.2 CHANGES IN PEROXIDE VALUE

The amount of peroxides were measured to be significantly (α=0.05) higher in the fortified fish oils at day 0, with the oil fortified with *Ulva* containing the highest concentration, see **Figure 24 (A)**. After 28 days of storage, there were 22% and 21% less peroxides (α=0.05) in the fish oil fortified with *Porphyra* and *Ulva*, see **Figure 24 (B)**.

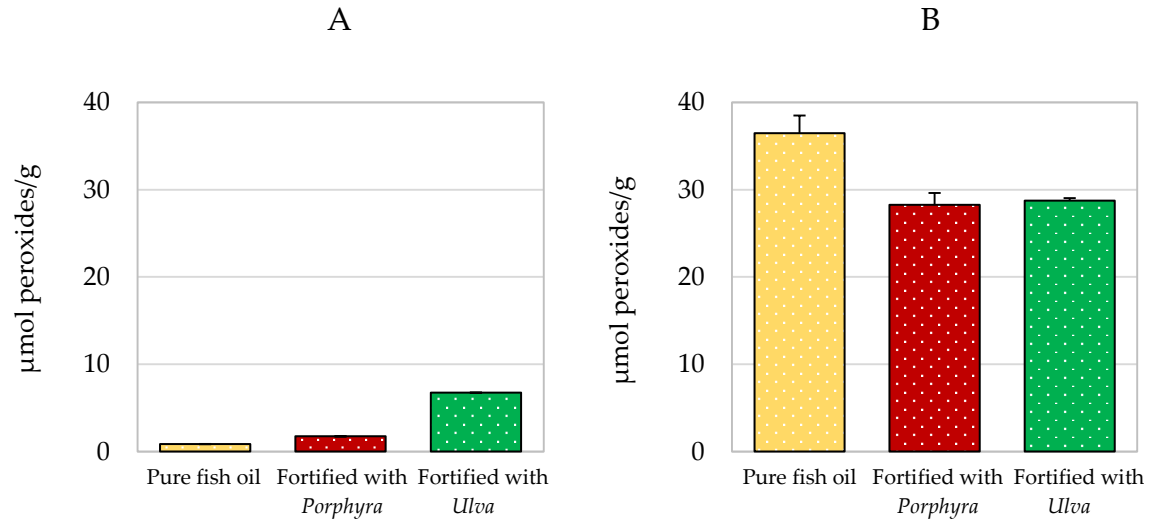

Figure 24. *Concentration of peroxides in the pure fish oil and fish oils fortified with Porhyra and Ulva at (A) day of fortification and (B) after 28 days of daylight storage at room temperature ± standard deviation (n=2)*

4.3.3.3 CHANGES IN REACTIVE ALDEHYDES

The amount of MDA, HHE and HNE produced in the fish oils during storage are shown in **Figure 25**. The concentration of MDA in the pure fish oil was significantly (α=0.05) higher compared to the fish oil fortified with both *Porphyra* and *Ulva* at day 7 and 28. The concentration of HHE and HNE in the pure fish oil was significantly (α=0.05) lower compared to the fish oil fortified with *Porphyra* at day 7, and the fish oil fortified with *Ulva* at day 28. The concentration of MDA was higher than HHE and HNE. The concentration of HHE, being formed from n-3 PUFA, was also higher compared to HNE, being formed from n-6 PUFA. When looking on the total amount of aldehydes, the fish oil fortified with *Ulva*, contained significantly (α=0.05) more aldehydes compared to the pure fish oil at day 0 (0,75nmol/g compared to 0,26nmol/g). However, after 7 days the fish oil fortified with *Ulva* contained significantly (α=0.05) less aldehydes compared to the pure fish oil (1,02nmol/g compared to 2,96nmol/g). The same trend was shown after 28 days when the concentration was 6,71nmol/g compared to 12,86nmol/g.

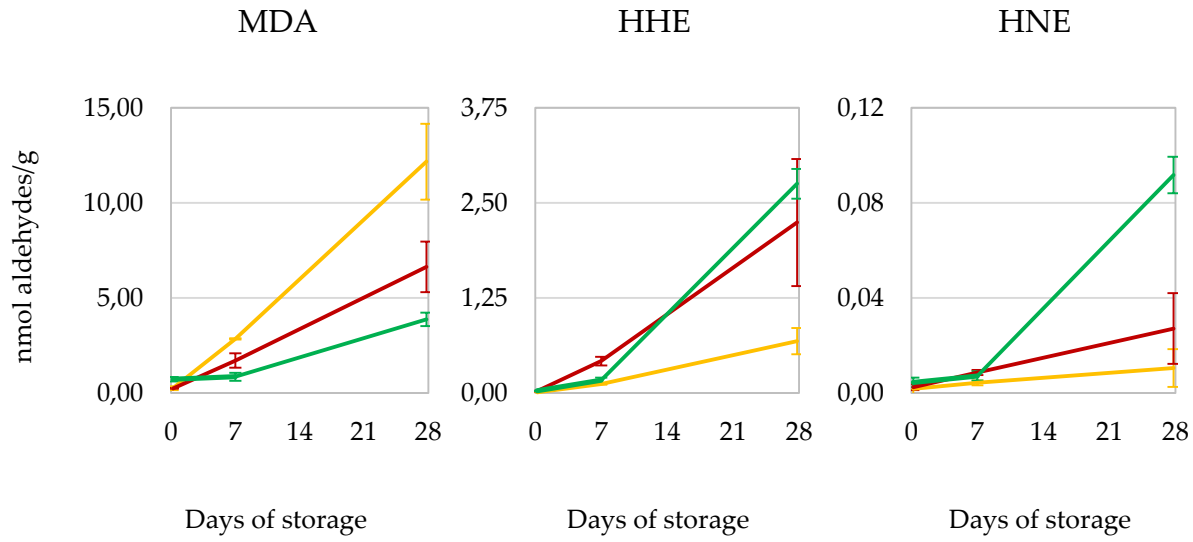

Figure 25. *Concentration of aldehydes (MDA, HHE and HNE) in the pure fish oil (yellow) and fish oils fortified with Porhyra (red) and Ulva (green) at day 0, 7 and following daylight storage at room temperature ± standard deviation (n=2)*

4.3.3.4 SENSORY ANALYSIS OF RANCID ODOR

The result from the sensory analysis of the pure and fortified fish oils stored in daylight at room temperature is shown in **Figure 26**. No significant (α=0.05) differences were detected, and no conclusions can be drawn from this analysis method. The sensory analysis for the pure and fortified fish oils stored in dark at room temperature and at 8˚C can be seen in **Appendix C**.

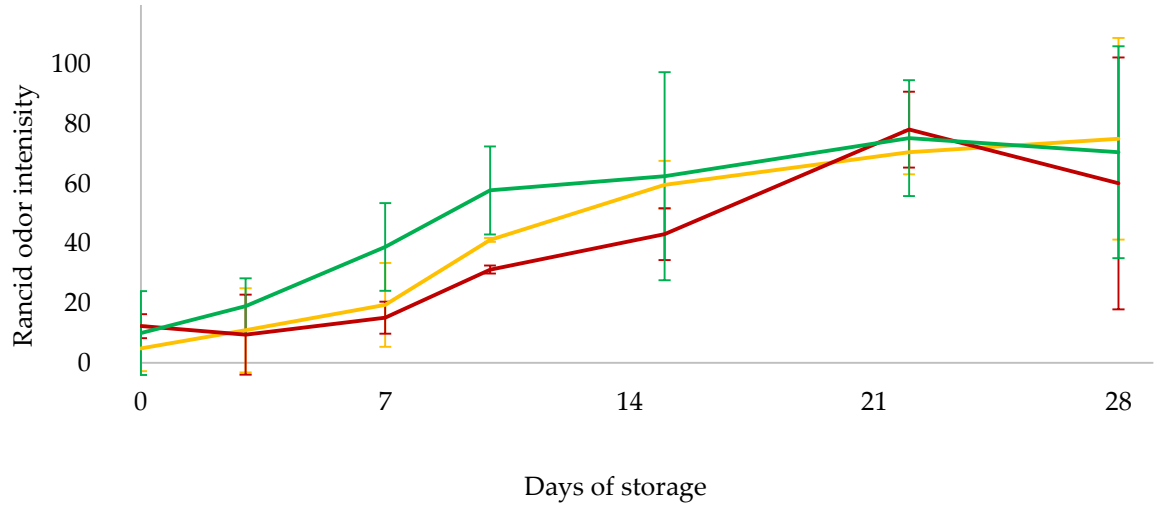

Figure 26. *Intensity of rancid odor in pure fish oil (yellow) and fish oil fortified with Porphyra (red) and Ulva (green) stored in daylight in room temperature for 0-28 days (n=2)*

4.3.4 LIPID OXIDATION DURING IN VITRO DIGESTION

The amount of reactive aldehydes produced in the intestinal step during the *in vitro* digestion are showed in **Figure 27**. When entering the intestinal stage and after 45 minutes, the amount of HHE was significantly (α=0.05) higher in the fish oil fortified with *Ulva*. This was also true for the amount of MDA after 45 minutes digestion of the fish oil fortified with *Ulva*. No HNE was detected in this analysis.

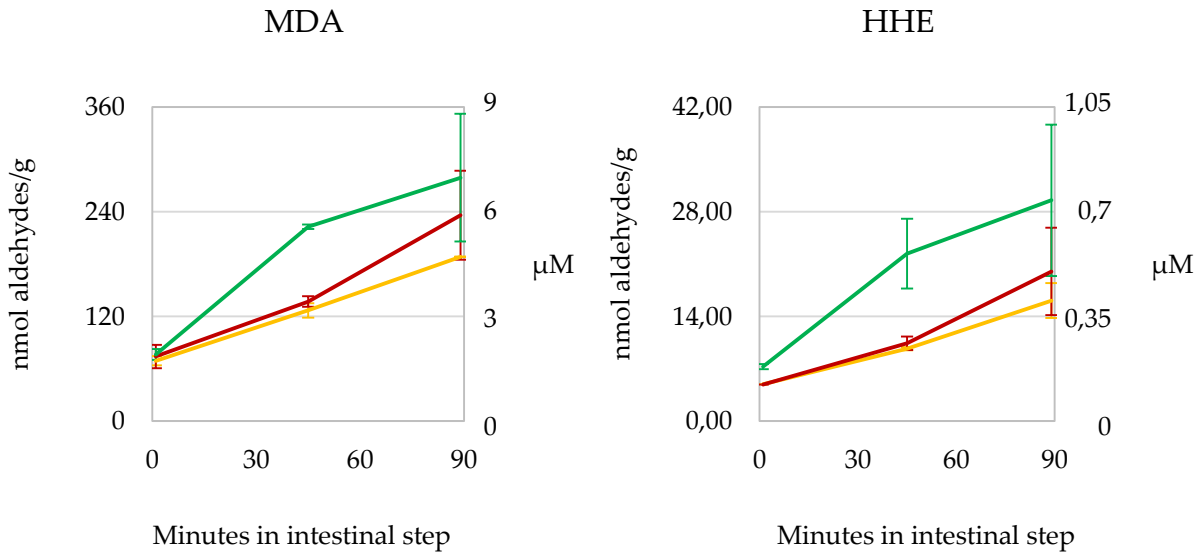

Figure 27. *Concentration of aldehydes (MDA and HHE) in the intestinal step of the in vitro digestion on pure fish oil (yellow) and fish oils fortified with Porhyra (red) and Ulva (green) ± standard deviation (n=2). Units are expressed as nmol aldehydes/g fish oil on the left y-axis, and µM aldehydes in the intestinal fluid on the right y-axis. Results from analysis of digestion blanks were subtracted from the oil containing digesta*

5 DISCUSSION

The fatty acid composition of the seaweed species shown in **Table 8** illustrates that the *Ulva* contain more SC fatty acids, and the *Porphyra* contain more LC fatty acids in contrast to one another. This relationship has also been previously reported by e.g. *Johns et al.* (1979). It was a strikingly high level of the LC n-3 PUFA EPA in Porphyra; ~42% of total fatty acids, making this seaweed highly interesting from a functional foods perspective. 100g dry *Porphyra* contained ~840mg EPA. The limit for functional health claims is set at 40mg EPA+DHA/100g food product according to EC Regulation 432/2012 [56].

Using the polytron at 24000rpm until the oil had reached 50˚C was considered the most successful method to extract seaweed biomass in sunflower oil. This was concluded due to the total change in color and the high amount of extracted carotenoids for both the *Ulva* and *Porphyra*. A more traditional approach might be to only look at the amount of phenolic compounds extracted. This is a compound group that is very famous for its capacity to inhibit lipid peroxidation. However, due to the method of analyzing phenolic compounds being imprecise, it was hard to conclude anything from the data generated. Instead it was assumed that the extraction of specific pigments and the change color indicated an overall higher lipophilic compound extraction, leading to more lipophilic phenolic compounds being extracted too. Having access to a LC/MS based method to measure the phenolic compounds might have led to a different extraction equipment being considered the most successful one.

Another incitement for choosing the polytron, is the possibility to upscale the process. There are larger industrial polytrons which can handle volumes up to 6000 liters. The method was also equally good at changing the total color and extracting lipophilic compounds from wet and dry seaweed, see **Table B.2** in **Appendix B**. From an economical point of view; use of wet biomass can be an advantage as drying costs are avoided. However, in the majority of this report, dry biomass was used as it was more easily handled and gave the ability to disintegrate it into smaller pieces, giving a more homogenous powder.

The polytron extraction method was not able to increase the concentration of the LC n-3 PUFAs EPA or DHA by extracting seaweed with sunflower oil. We had expected that the *Porphyra*, containing 42% of its total fatty acids as EPA, would have been able to provide a certain enrichment with this fatty acid. A small but significant increase was however noticed in the concentration of C17:1 n7 and ALA. Possibly, the LC n-3 PUFA are firmly bound in lipid classes such as glycolipids or phospholipids that are difficult to extract with such a hydrophobic media as oil [57].

By comparing the fortified fish oils stored in daylight at room temperature to the fortified fish oils stored in darkness at 8˚C, shown in **Figure 22** and **Appendix C**, it is evident that a pellet formed as the oils were bleached. The bleaching is most likely due

to oxidation of pigments, resulting in loss of conjugated structure. In the case of chlorophyll, the oxidation also results in loss of its porphyrin structure. The structural change makes the chlorophyll precipitate, as it looses its coordiantion to magnesium [58]. This precipitation might explanain the aformentioned pellet.

The fish oil fortified with *Porphyra* and *Ulva* contained no more ALA, EPA or DHA compared to pure fish oil, at the day of fortification (day 0). The fact that the fortified fish oils contained less total fatty acids can be due to a dilution effect of other fat soluble compounds extracted from the seaweed, such as antioxidants. After 28 days of daylight storage at room temperature had passed, the fish oil fortified with *Ulva* contained more ALA, EPA and DHA, see **Table 11**. This suggests that antioxidants from the *Ulva* managed to stabilize the fatty acids in fish oil. For *Porphyra*-enriched fish oils, this was true for EPA and DHA, see **Table 12** and **Figure 23.** Earlier studies have shown that methanol-extracts from *Porphyra* and *Ulva* have managed to stabilize fish oil during storage [59, 60].

The peroxide value was significantly (α=0.05) higher for the fish oil fortified with *Porphyra* and especially *Ulva,* see **Figure 24 (A)**. A study performed by *Gao and Kispert* (2003) showed that carotenoids can abstract an electron from ferrous iron, yielding ferric iron [61]. This is the ion which reacts with thiocyanate to indicate the content of peroxides, meaning that carotenoids might interfere with the measurement of PV. It is hence likely that the level of peroxidation is overestimated in the fortified oils after 0 days of storage, where the concentrations of carotenoids are high, see **Figure 21**. However, as the carotenoid concentration is not proportionally higher in the fish oil fortified with *Ulva* compared to the fish oil fortified with *Porphyra*, there might be more compounds interfering with the peroxide value analysis. During time, carotenoids are expected to be degraded by light and oxidized in order to preserve the fish oil. The carotenoids would then no longer interfere. This is in line with what we see in **Figure 24 (B)**.

The concentration of MDA in the storage study was higher in the pure fish oil at day 7 and 28, compared to both fortified fish oils. This proved that seaweed compounds extracted into fish oils had some effect on the production of secondary oxidation products. However, when looking at the production of HNE and HHE, these aldehydes were higher in the fortified oils compared to the pure fish oil. This points towards the seaweed promoting oxidation reaction pathways yielding HHE and HNE.

Balancing together chemical and sensory analyses of lipid oxidation, the effect from lipophilic seaweed compounds in protecting fish oil was limited. Possibly aqueous seaweed compounds are more antioxidative towards fish oil, and would give better protection. This has been suggested by *Porter* (1993) as the polar paradox [62]. The paradox suggests that water-soluble antioxidants are better in oil-based systems, than oil-soluble antioxidants. This can be explained by taking into account that lipid oxidation is prominent on the surface layer of the oil. If the oil system contains water-

soluble antioxidants, the antioxidants would be oriented on the air-oil interface. This means that the antioxidants are located where they are mostly needed. This reverse relationship have been confirmed by *Frankel* (1994) [63].

In a second storage study, this master's thesis project screened whether the Swedish brown seaweed species *Saccharina latissima* and *Fucus vesiculosus* were able to preserve the fish oil more successfully against development of rancid odor, MDA, HHE and HNE. According to the literature, these brown seaweed species contain more phenolic compounds compared to *Porphyra* and *Ulva* [45]. Fish oil fortified with the brown species of seaweed followed the same trend as the fish oils fortified with *Porphyra* and *Ulva* in part 4.3.3.3 and 4.3.3.4. The seaweed extraction inhibited the production of MDA, but promoted the production of both HHE and HNE. All oils were in general more oxidized in this second storage study, perhaps due to the increased amount of daylight as this study was conducted during the spring in Sweden. The sensory analysis was also not able to detect any differences in rancid odor between fortified and pure fish oils. The results are presented in **Appendix E**.

In the *in vitro* digestion, the use of gastric lipase is not recommended at this time according to the Infogest-protocol. However, as our focus is on lipids, it was desirable to include gastric lipase. The concentration of lipase in the SGF was based on findings on human gastric lipase concentration from *Tullberg et al.* (2016) [31]. This study measured the activity of lipase in human gastric juices to 16U/ml [31]. As human gastric lipase is not commercially available, lipase from *Rhizopus oryzae* was used instead. Lipase from *Rhizopus oryzae* was compared to recombinant human gastric lipase in a long chain triacylglyceride formula in a study by *Sassene et al.* (2016) [51]. The lipase from *Rhizopus oryzae* released twice the amount of fatty acids compared to recombinant human gastric lipase after 60 minutes of digestion [51]. This lead the concentration of lipase from *Rhizopus oryzae* in the SGF to be 8U/ml.

The aldehyde concentration at the beginning of the intestinal step was ~75nmol MDA/g fish oil or ~1.8µM MDA in the digestive fluid. This value is higher compared to what was seen in the pure fish oil stored 28 days in daylight at room temperature (12.6nmol MDA/g fish oil, see **Figure 25**). It points towards lipid peroxidation being promoted in the gastric step, where the oil is still as bulk or large droplets floating on top of the water phase. There are lipases active in the simulated gastric fluid. With a low pH, and 37˚C, this leads to some degradation of triglycerides along the surface of the droplet. The fish oil fortified with *Ulva* contained more HHE in the beginning of the intestinal step compared to pure fish oil. When comparing our initial concentration of MDA in pure fish oil against previously reported concentrations by *Larsson et al.* (2012) (~25nmol TBARS/g pre-emulsified fish oil) and *Tullberg et al.* (2016) using their porcine model II (<0.2µM MDA/g pre-emulsified fish oil), it points towards lipid peroxidation being more promoted in our gastric step [31, 54]. The reason for this is unknown. A possible explanation is that the digestion blanks which were subtracted from the fish oil containing digesta were not prepared on the same day as the samples.

It is therefore possible that the digestion differed in some small, but significant way. For example, the heat during incubation could differ by some degree, giving a different activity of the enzymes. Regardless of absolute values, the difference between the pure and fortified fish oils remains. After 45 minutes in the intestinal step, the fish oil fortified with *Ulva* kept promoting lipid peroxidation as the amount of MDA and HHE were higher compared to the pure fish oil. In the intestinal step, the oil is emulsified in the water phase, giving a larger total surface area. This leads to triglycerides being further degraded, and free fatty acids plus monoglycerides being further exposed to tentative prooxidants, promoting lipid peroxidation. When looking at the concentration of MDA, HHE and HNE in the intestinal fluid, it should be noted that the concentrations reached were far from the reported LC50 value for endothelial cells [33].

There might be other positive aspects of a vegetable oil fortified with seaweed than a stabilization effect. The Swedish company *AB Gastronova* extract the umami flavor from seaweed grown along the west coast of Sweden. They use the extract to fortify e.g. rapeseed and olive oil to produce food products under the name *Seamami®*. Except for the umami taste, the company claims that their product contains an increased amount of vitamins and trace metals elevating the nutritional value. Furthermore, the trend aspect of using seaweed in food is strong at the moment.

6 CONCLUSION

The most successful method to extract lipophilic compounds (pigments and phenolic compounds) from *Porphyra* and *Ulva* into sunflower oil was the polytron-based method. No increase in LC n-3 PUFA was however detected when extracting the seaweed into sunflower oil using this method. Therefore the first hypothesis "*Seaweed can enrich vegetable oils with LC n-3 PUFAs"* was not proven by this study.

By extracting *Porphyra* and *Ulva* with fish oil, we successfully preserved the amount of ALA, EPA and DHA during daylight storage at room temperature for 28 days. The amount of peroxides and MDA produced were also less in the fortified fish oils compared to the pure fish oil. However, the amount of HHE and HNE was higher in the fortified fish oils. The sensory study of rancid odor did not manage to differentiate the oils. The *in vitro* digestion pointed towards the fortified oils promoting lipid peroxidation in the intestinal tract. Thereby, the second hypothesis *"Seaweed antioxidants have a preservative effect on fish oil"* was only proven to a small extent.

6.1 FUTURE WORK

It would be interesting to look more specifically into which compounds from the seaweed that transfers to the fish oil when fortified. From this study we have used general spectrophotometric methods to determine the concentration of carotenoids and phenolic compounds. A more precise measure of these compound groups using chromatography-based methods, could identify the carotenoids and phenolic compounds further. When it comes to the prooxidants, we only measured chlorophyll, using the aforementioned spectrophotometric method. There are probably other prooxidants such as trace metals which also promotes lipid peroxidation. This could be tested by adding a chelator such as EDTA to another set of fortified oils, and see if the oils were preserved better. Another group of compounds that would be interesting to analyze is the one that interfere with the analysis of phenolic compounds and peroxide value. When measuring phenolic compounds, it happened quite often that the sunflower oils fortified with seaweed appeard to contain less phenolic compounds compared to pure sunflower oil. This suggests that there are compounds in the seaweed scavenging phenolic compounds.

Another method of extraction of lipophilic compounds from seaweed could be used. The four methods we tried, were chosen due to equipment being available at the *Division of Food and Nutrition Science*. Using supercritical fluid extraction with ethanol as a co-solvent on freeze dried ground seaweed would possibly extract much more of the lipid phase. Furthermore it would also extract some hydrophilic compounds. By the addition of an emulsifier, hydrophilic antioxidants, such as the vast majority of phenolic compounds, could be solubilized into the extracted lipid phase. This phase could then be added to fortify food.

The seaweed could also in a future perspective be used to fortify other types of vegetable oil and fish oil. As different oils have different polarity, they are more or less likely to dissolve LC n-3 PUFA and seaweed antioxidants. Other seaweed species could also be extracted and evaluated based on their ability to fortify vegetable and fish oils with LC n-3 PUFA and antioxidants.

In the *in vitro* digestion, as we got higher concentrations of aldehydes at the beginning of our intestinal step than previously reported. It would be interesting to analyze when during the gastric step the concentration of aldehydes started to increase. This analysis would answer if the aldehyde production is linked to e.g. the addition of SGF or when the pH decreased from 6 to 3.

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CHALMERS
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APPENDICES

Extraction of a seaweed lipid fraction and evaluation of its ability to prevent lipid peroxidation in fish oil

Master's thesis in Biology and Biological Engineering

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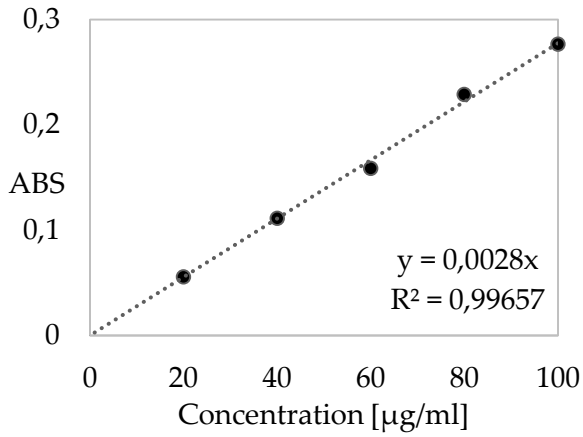
APPENDIX A

STANDARD CURVES

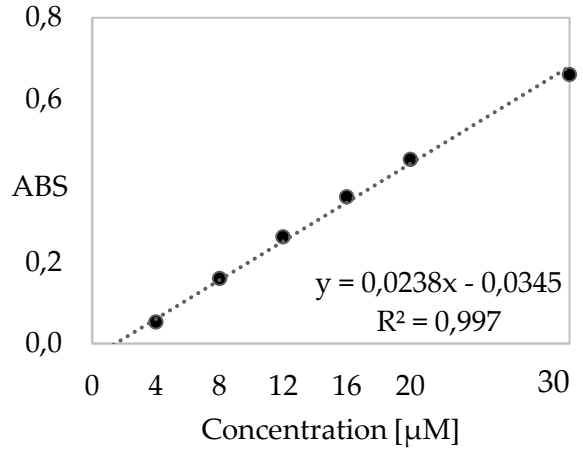
PHENOLIC COMPOUNDS



The standard curve for phloroglucinol was created as described in part 3.1.4.2. See **Figure A.1** for result. The trend line was forced through the origin, giving an R^2 equal to 0.99657 and an equation where Y = 0.0028X

Figure A.1. *Standard curve for phloro-glucinol in the range of 0-100 µg/ml recorded at 765nm*

PEROXIDE VALUE



The standard curve for cumene hydroperoxide (CPO) was created as described in part 3.3.1.1. See **Figure A.2** for result. The trend line was not forced through the origin, giving an R^2 equal to 0.99696 and an equation where Y = 0.0238X - 0.0345.

Figure A.2. *Standard curve for cumene hydroperoxide in the range 0-30µM recorded at 500nm*

APPENDIX B

FORTIFICATION OF SUNFLOWER OIL – OPTIMIZATION OF EXTRACTION METHODS

POLYTRON

The experimental setup for the polytron can be seen in **Table B.1**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.1**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.2**. Statistical data (F-values) for the duplicate run can be seen in **Table B.2**.

Table B.1. *The 2^4-factorial design used for testing the polytron (same as **Table 2**)*

	Specie	State	Time [min]	Freq. [rpm]
0	0	0	1	14000
1	*Porphyra*	Dry	1	14000
2	*Ulva*	Dry	1	14000
3	*Porphyra*	Wet	1	14000
4	*Ulva*	Wet	1	14000
5	*Porphyra*	Dry	2	14000
6	*Ulva*	Dry	2	14000
7	*Porphyra*	Wet	2	14000
8	*Ulva*	Wet	2	14000
9	*Porphyra*	Dry	1	24000
10	*Ulva*	Dry	1	24000
11	*Porphyra*	Wet	1	24000
12	*Ulva*	Wet	1	24000
13	*Porphyra*	Dry	2	24000
14	*Ulva*	Dry	2	24000
15	*Porphyra*	Wet	2	24000
16	*Ulva*	Wet	2	24000

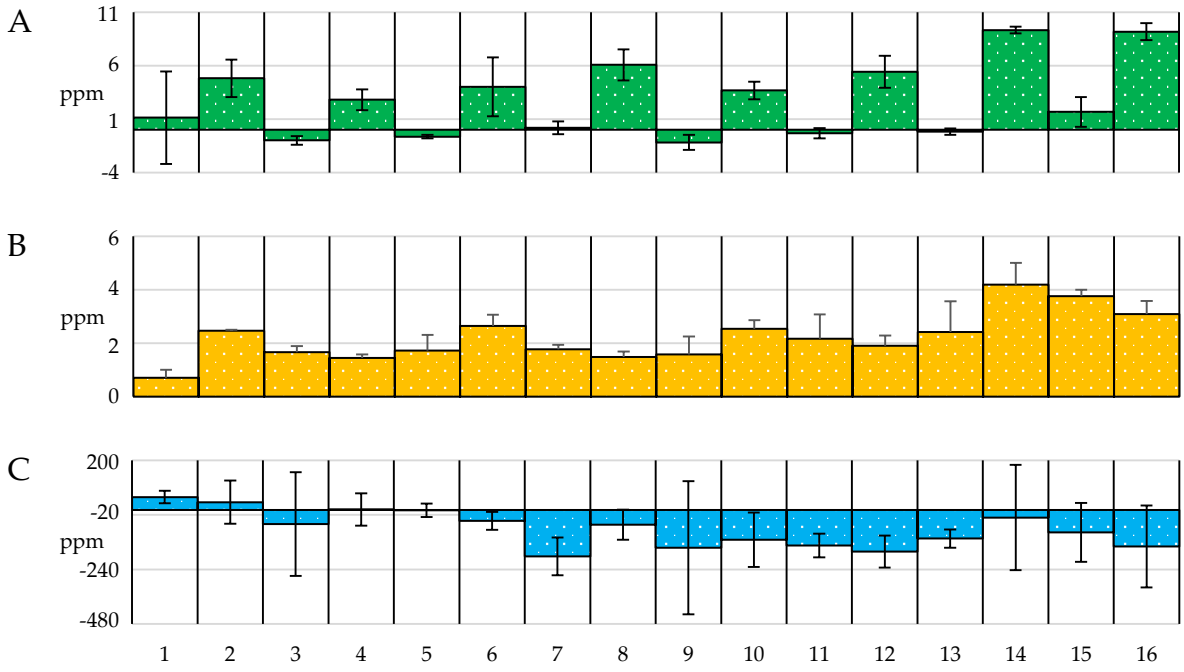


Figure B.1. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted in sunflower oil when using the polytron ± standard deviation (n=2). The sample numbers are explained in **Table B.1***

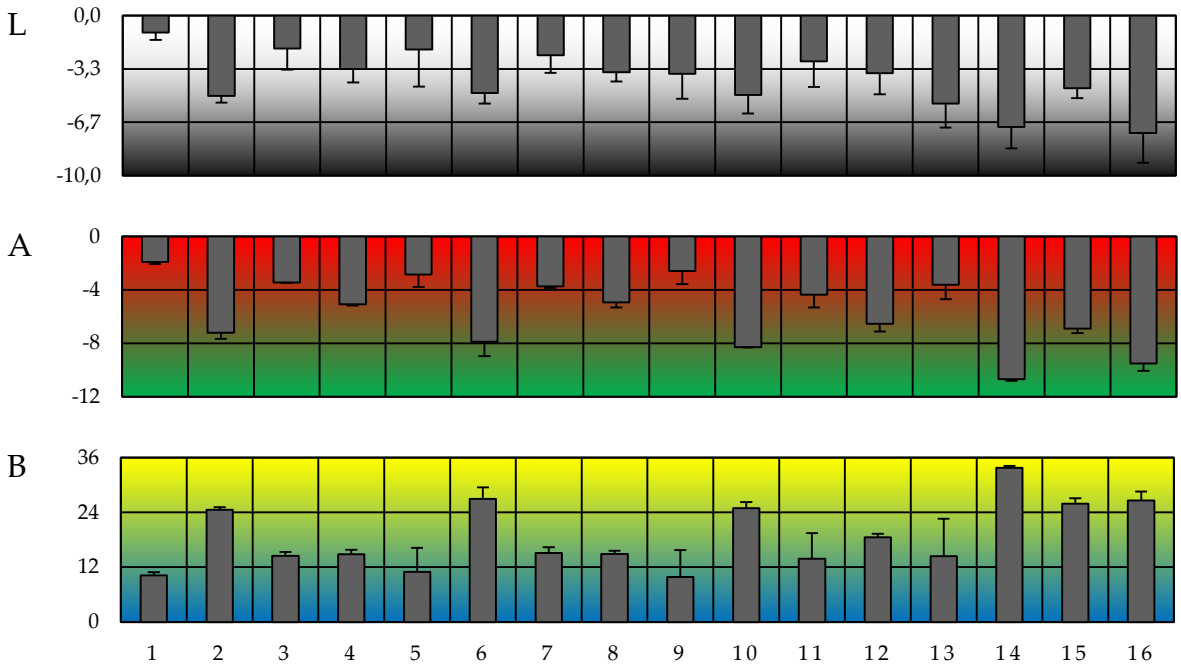


Figure B.2. *L-, a- or b-value for sunflower oil when seaweed was extracted with polytron ± standard deviation (n=2). The sample numbers are explained in* ***Table B.1***

Table B.2. *Statistical data (F-values) of all single effect parameters for the polytron run in a duplicate according to the settings showed in* ***Table B.1****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**Specie**	**State**	**Time**	**Power**	***Model***
Chlorophyll A	209.15	8.14	10.83	8.52	*26.63*
Chlorophyll B	36.25	0.74	7.34	2.43	*6.19*
Total Chlorophyll	105.55	0.46	10.35	5.29	*13.99*
Carotenoids	7.49	0.46	20.32	28.00	*8.69*
Phenolic Compounds	0.25	2.11	0.14	4.71	*1.05*
Total change in color	**Specie**	**State**	**Time**	**Power**	***Model***
L	18.86	1.50	9.38	17.91	*5.43*
A	283.25	0.08	34.08	71.43	*48.97*
B	37.06	2.66	20.25	32.93	*30.63*
Whiteness	45.31	3.73	22.48	38.87	*22.76*

Significance
$\alpha = 0.05$ F = 4.32
$\alpha = 0.01$ F = 8.02
$\alpha = 0.001$ F = 14.59

BEADBEATER

The experimental setup for the beadbeater can be seen in **Table B.3**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.3**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.4**. Statistical data (F-values) for the duplicate run can be seen in **Table B.4**. Statistical data (F-values) for the second run can be seen in **Table B.5**.

Table B.3. *The 2^4-factorial design used for testing the beadbeater (same as **Table 3**)*

	Specie	State	Time [min]	Freq. [rpm]
0	0	0	1	1200
1	*Porphyra*	Dry	1	1200
2	*Ulva*	Dry	1	1200
3	*Porphyra*	Wet	1	1200
4	*Ulva*	Wet	1	1200
5	*Porphyra*	Dry	5	1200
6	*Ulva*	Dry	5	1200
7	*Porphyra*	Wet	5	1200
8	*Ulva*	Wet	5	1200
9	*Porphyra*	Dry	1	1800
10	*Ulva*	Dry	1	1800
11	*Porphyra*	Wet	1	1800
12	*Ulva*	Wet	1	1800
13	*Porphyra*	Dry	5	1800
14	*Ulva*	Dry	5	1800
15	*Porphyra*	Wet	5	1800
16	*Ulva*	Wet	5	1800

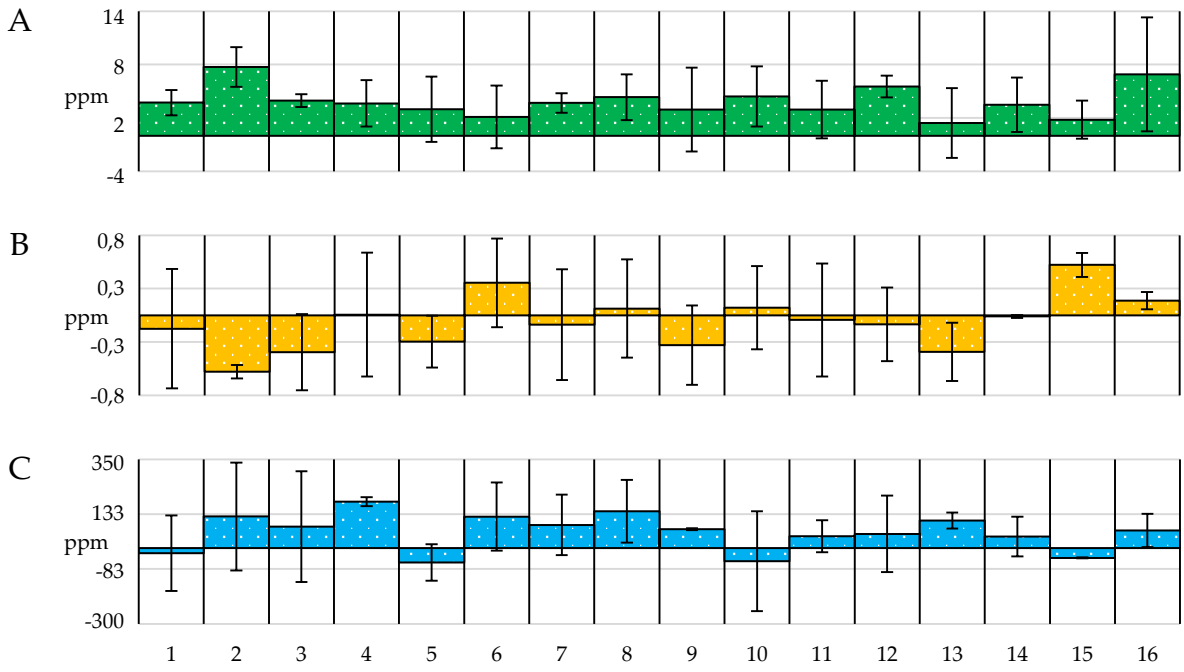


Figure B.3. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted in sunflower oil when using the beadbeater ± standard deviation (n=2). The sample numbers are explained in **Table B.4***

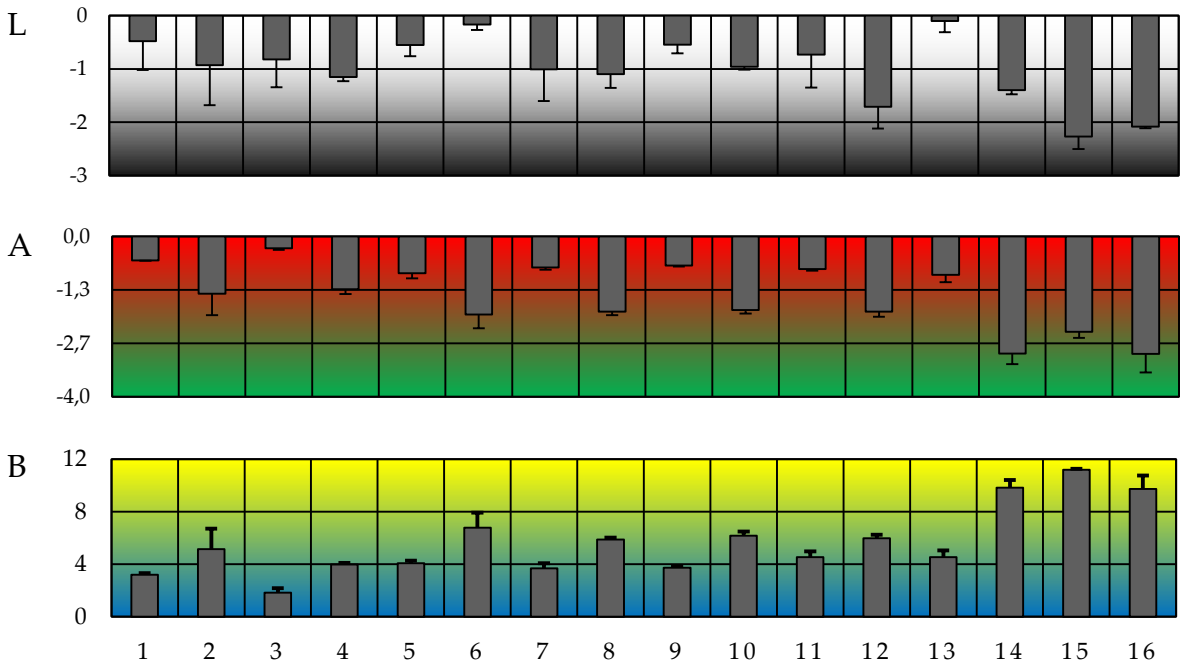


Figure B.4. *L-, a- or b-value for sunflower oil when seaweed was extracted with beadbeater ± standard deviation (n=2). The sample numbers are explained in* ***Table B.4***

Table B.4. *Statistical data (F-values) of all single effect parameters for the beadbeater run in a duplicate according to the settings showed in* ***Table B.3.*** *The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**Specie**	**State**	**Time**	**Power**	***Model***
Chlorophyll A	8.96	1.91	0.45	0.00	*1.70*
Chlorophyll B	0.96	0.01	1.28	0.24	*0.46*
Total Chlorophyll	3.00	0.22	0.93	0.10	*0.70*
Carotenoids	0.75	1.33	2.13	0.63	*0.64*
Phenolic Compounds	1.53	0.78	0.00	1.24	*0.85*
Total change in color	**Specie**	**State**	**Time**	**Power**	***Model***
L	6.13	22.59	1.23	8.79	*5.45*
A	109.60	1.24	49.36	40.80	*22.07*
B	30.22	1.22	48.41	48.18	*16.20*
Whiteness	18.30	11.89	20.52	29.80	*10.93*

Significance
$\alpha = 0.05$
F = 4.32
$\alpha = 0.01$
F = 8.02
$\alpha = 0.001$
F = 14.59

Table B.5. *Statistical data (F-values) of all single effect parameters for the beadbeater in a single run according to the settings showed in* ***Table B.3****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**Specie**	**State**	**Time**	**Power**	***Model***
Chlorophyll A	10.71	2.34	0.15	1.42	*2.46*
Chlorophyll B	1.61	0.12	0.10	1.15	*0.69*
Total Chlorophyll	4.43	0.19	0.00	1.26	*1.16*
Carotenoids	2.24	0.21	13.39	7.01	*2.82*
Phenolic Compounds	0.99	0.07	0.36	15.11	*2.53*
Total change in color	**Specie**	**State**	**Time**	**Power**	***Model***
L	4.17	7.02	0.69	6.32	*2.81*
A	77.01	0.11	31.91	23.01	*14.86*
B	18.37	0.00	22.43	20.14	*7.93*
Whiteness	8.94	1.98	7.76	11.36	*4.08*

Significance
$\alpha = 0.05$ F = 6.61
$\alpha = 0.01$ F = 16.26
$\alpha = 0.001$ F = 47.18

SONICATOR BATH

The experimental setup for the sonicator bath can be seen in **Table B.6**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.5**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.6**. Statistical data (F-values) for the single run can be seen in **Table B.7**.

Table B.6. *The 2^3-factorial design used for testing the sonicator bath (same as* ***Table 4****)*

	Specie	State	Time [min]
0	0	0	1
1	*Porphyra*	Dry	1
2	*Ulva*	Dry	1
3	*Porphyra*	Wet	1
4	*Ulva*	Wet	1
5	*Porphyra*	Dry	5
6	*Ulva*	Dry	5
7	*Porphyra*	Wet	5
8	*Ulva*	Wet	5

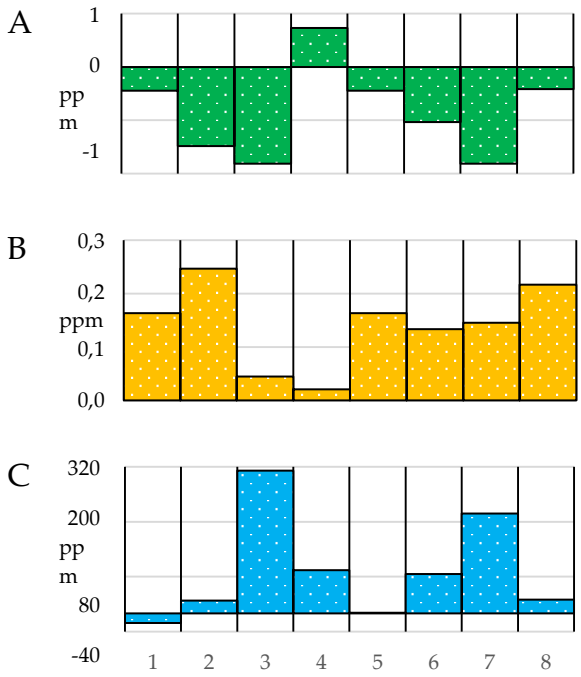


Figure B.5. *Concentration of (A) chlorophyll, (B) carotenoids and (C) total phenolic compounds extracted in sunflower oil when using the sonicator bath. The sample numbers are explained in* ***Table B.6***

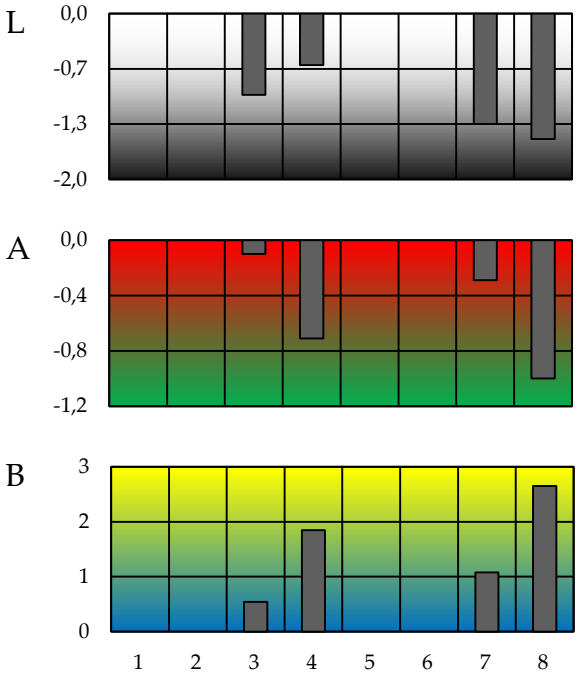


Figure B.6. *L-, a- or b-value for sunflower oil when seaweed was extracted with sonicator bath. The sample numbers are explained in* ***Table B.6***

Table B.7. *Statistical data (F-values) of all single effect parameters for the sonicator bath in a single run according to the settings showed in* ***Table B.6****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**Specie**	**State**	**Time**	***Model***
Chlorophyll A	26.59	3.09	0.30	*21.35*
Chlorophyll B	0.37	0.10	0.17	*1.00*
Total Chlorophyll	2.10	0.00	0.19	*2.60*
Carotenoids	0.24	1.81	0.79	*1.12*
Phenolic Compounds	1983	8388	160	*3324*
Total change in color	**Specie**	**State**	**Time**	***Model***
L	0.11	-	5.27	*13.23*
A	174.24	-	23.04	*139.43*
B	122.70	-	26.56	*142.26*
Whiteness	0.42	-	5.74	*15.06*

Significance
$\alpha = 0.05$ F = 161.45
$\alpha = 0.01$ F = 4052.18
$\alpha = 0.001$ F = 405284.07

SONICATOR PROBE

TEST 1

The experimental setup for the first test of the sonicator probe can be seen in **Figure B.7**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.8**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.9**. Statistical data (F-values) can be seen in **Table B.8**.

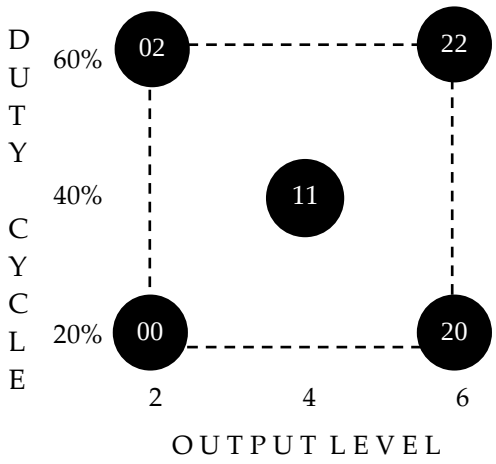


Figure B.7. *The 2^2-factorial design with 3 center points used to evaluate the sonicator probe (same as **Figure 5**)*

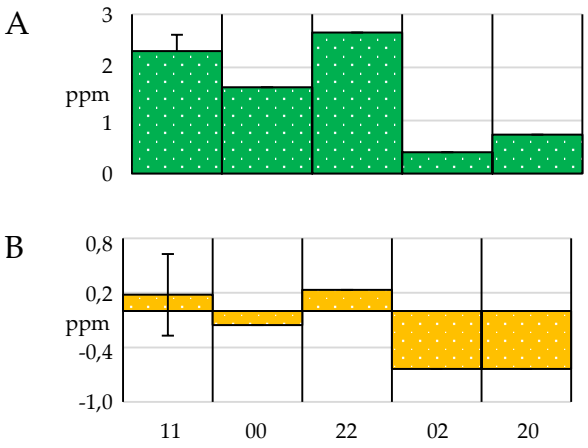


Figure B.8. *Concentration of (A) chlorophyll and (B) carotenoids extracted in sunflower oil when using the sonicator probe ± standard deviation for sample number 11 (n=3). The sample numbers are explained in Figure B.7*

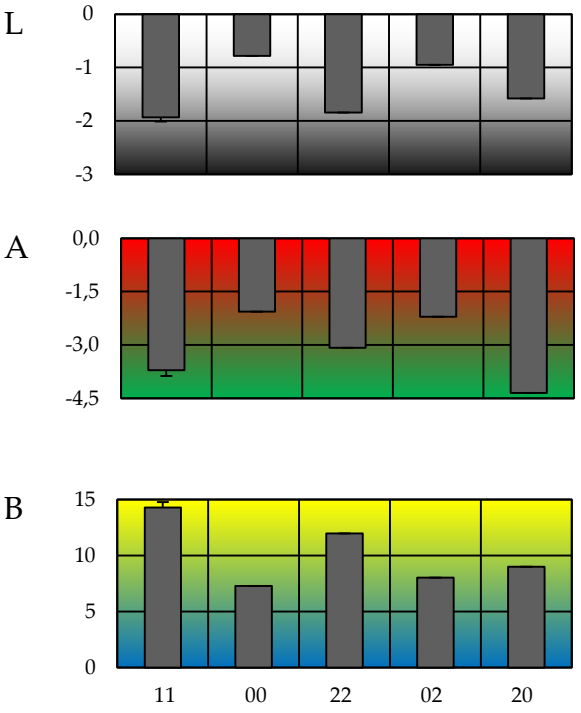


Figure B.9. *L-, a- or b-value for sunflower oil when seaweed was extracted with sonicator probe ± standard deviation för sample number 11 (n=3). The sample numbers are explained in **Figure B.7***

Table B.8. *Statistical data (F-values) of all single and combined effect parameters for the sonicator probe according to the settings showed in* ***Table B.7****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**D.C.**	**O.L.**	**Comb.**	**Pure Quad.**	***Model***
Chlorophyll A	5.73	35.03	65.36	118.70	*35.38*
Chlorophyll B	0.43	1.05	12.08	3.49	*4.52*
Total Chlorophyll	1.33	5.02	26.81	16.90	*11.06*
Carotenoids	0.19	0.19	2.28	1.94	*0.89*
Phenolic Compounds	0.02	0.03	2.14	0.01	*0.73*
Total change in color	**D.C.**	**O.L.**	**Comb.**	**Pure Quad.**	***Model***
L	7.64	118.02	0.33	118.80	*42.00*
A	13.01	103.59	20.92	43.83	*45.84*
B	15.26	34.58	5.32	201.69	*18.39*
Whiteness	15.27	84.61	3.75	274.88	*34.55*

Significance
$\alpha = 0.05$ F = 18.51
$\alpha = 0.01$ F = 98.50
$\alpha = 0.001$ F = 998.50

SONICATOR PROBE

TEST 2

The experimental setup for the second test of the sonicator probe can be seen in **Figure B.10**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.11**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.12**. Statistical data (F-values) can be seen in **Table B.9**.

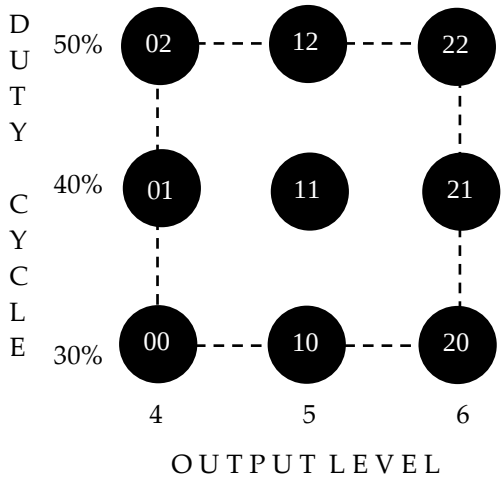


Figure B.10. The 3^2-factorial design used in the second evaluation of the sonicator probe *(same as* ***Figure 6****)*

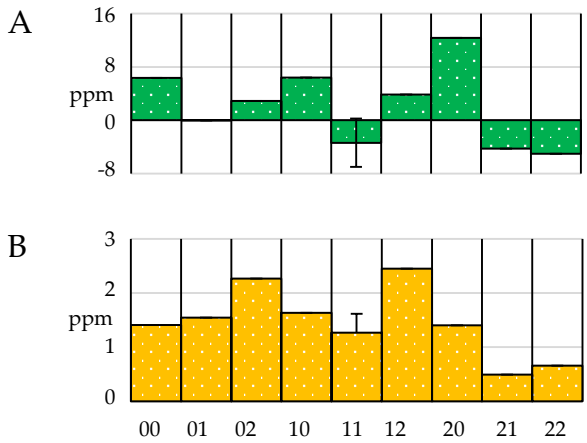
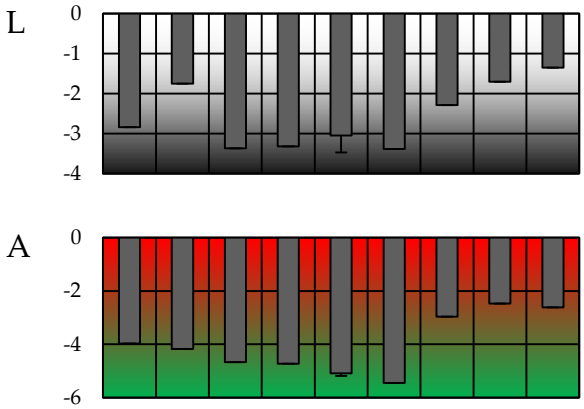


Figure B.11. *Concentration of (A) chlorophyll and (B) carotenoids extracted in sunflower oil when using the sonicator probe ± standard deviation for #11 (n=3). The sample numbers are explained in* ***Figure B.10***



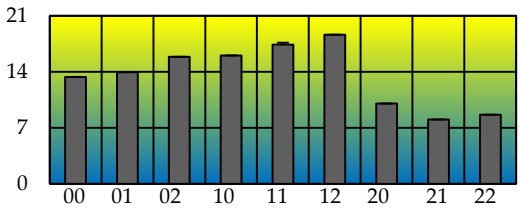


Figure B.12. *L-, a- or b-value for sunflower oil when seaweed was extracted with sonicator probe ± standard deviation for #11 (n=3). The sample numbers are explained in* ***Figure B.10***

Table B.9. *Statistical data (F-values) of all single and combined effect parameters for the sonicator probe according to the settings showed in* ***Table B.10****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**D.C.**	**O.L.**	**Comb.**	***Model***
Chlorophyll A	0.00	0.39	5.01	*2.60*
Chlorophyll B	7.25	0.20	1.45	*2.59*
Total Chlorophyll	7.25	0.25	1.42	*2.59*
Carotenoids	2.95	6.89	1.89	*3.41*
Total change in color	**D.C.**	**O.L.**	**Comb.**	***Model***
L	2.04	9.22	1.63	*3.63*
A	10.78	400.48	9.16	*107.40*
B	22.07	774.27	20.45	*209.31*
Whiteness	4.15	107.92	4.19	*30.11*

Significance
$\alpha = 0.05$ F = 19.00
$\alpha = 0.01$ F = 99.00
$\alpha = 0.001$ F = 999.00

SONICATOR PROBE

TEST 3

For the third test when the duty cycle was allowed to spread between 40-80% on output level 5. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.13**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.14**. Statistical analysis (two sample t-test assuming equal variance) was done without being able to explain any significant (α=0.05) difference.

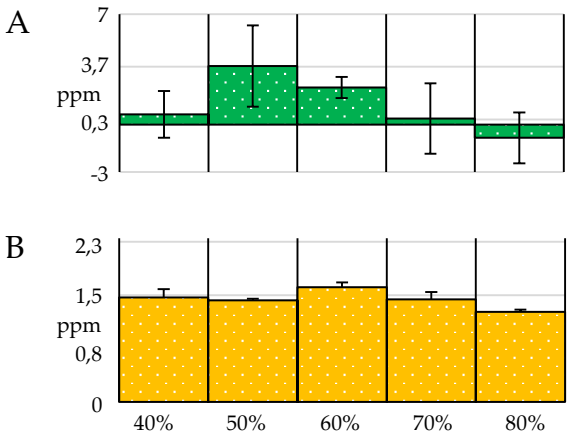


Figure B.13. *Concentration of (A) chlorophyll and (B) carotenoids extracted in sunflower oil when using the sonicator probe ± standard deviation (n=2)*

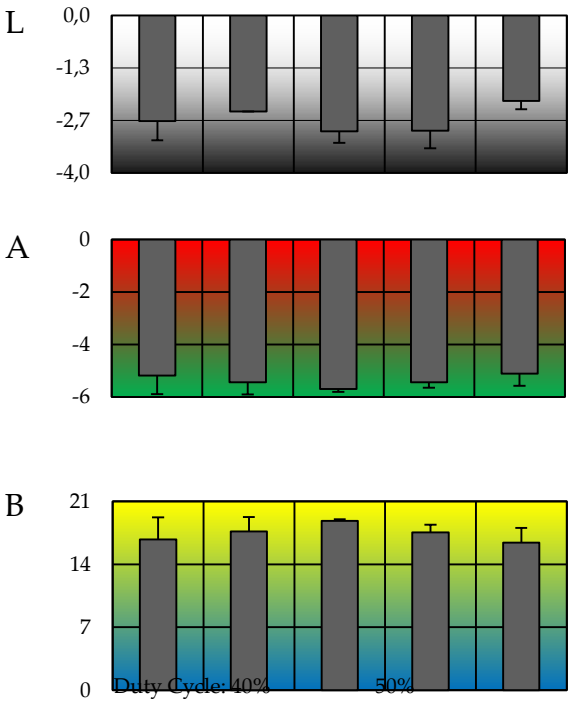


Figure B.14. *L-, a- or b-value for sunflower oil when seaweed was extracted with sonicator probe ± standard deviation (n=2)*

SONICATOR PROBE

TEST 4

The experimental setup for the fourth test of the sonicator probe can be seen in **Figure B.15**. The abundance of lipophilic compounds extracted from seaweed in sunflower oil is shown in **Figure B.16**. The total change in color according to the Hunter L, a, b color scale is shown in **Figure B.17**. Statistical data (F-values) can be seen in **Table B.10**.

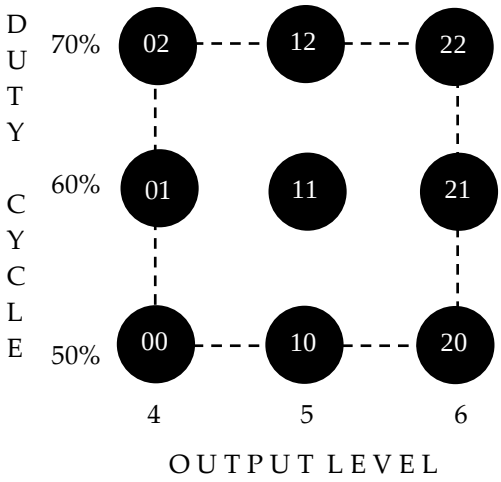


Figure B.15. *The 3^2-factorial design used in the fourth evaluation of the sonicator probe (same as **Figure 7**)*

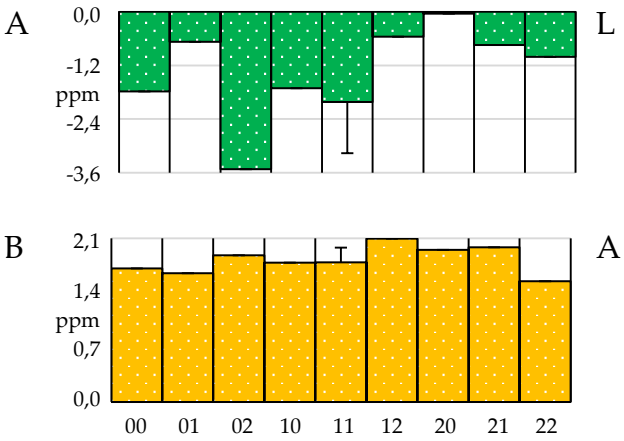


Figure B.16. *Concentration of (A) chlorophyll and (B) carotenoids extracted in sunflower oil when using the sonicator probe ± standard deviation for #11 (n=3). The sample numbers are explained in **Figure B.15***

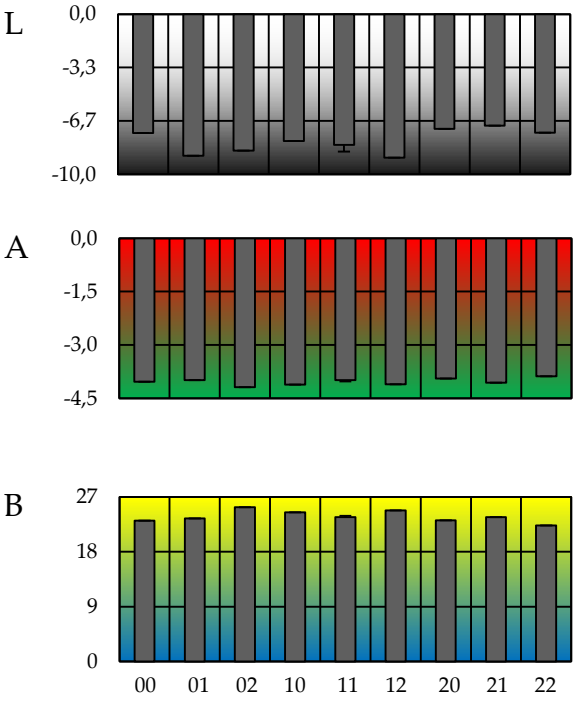


Figure B.17. *L-, a- or b-value for sunflower oil when seaweed is extracted with sonicator probe ± standard deviation for #11 (n=3). The sample numbers are explained in **Figure B.15***

Table B.10. *Statistical data (F-values) of all single and combined effect parameters for the sonicator probe according to the settings showed in* ***Table B.15****. The level of significance is color coded according to the right hand side of the table*

Lipophilic compounds	**D.C.**	**O.L.**	**Comb.**	***Model***
Chlorophyll A	0.25	0.66	0.71	*0.58*
Chlorophyll B	0.20	1.47	1.22	*1.03*
Total Chlorophyll	0.22	1.13	1.00	*0.83*
Carotenoids	0.02	0.44	1.49	*0.86*
Total change in color	**D.C.**	**O.L.**	**Comb.**	***Model***
L	2.59	6.76	1.11	*2.89*
A	0.84	6.82	6.74	*5.29*
B	6.07	26.01	20.05	*18.04*
Whiteness	4.82	17.27	4.36	*7.70*

Significance
$\alpha = 0.05$ F = 19.00
$\alpha = 0.01$ F = 99.00
$\alpha = 0.001$ F = 999.00

FATTY ACID COMPOSITION OF PURE AND FORTIFIED SUNFLOWER OIL

A two sample t-test assuming equal variance was preformed to compare the difference of each fatty acid between pure sunflower oil against sunflower oil fortified with *Porphyra* and *Ulva*. Statistical data comparing the fatty acid profile in **Table 9** can be seen in **Table B.11**. If the t-value is significant ($\alpha \leqslant 0.05$) and positive/negative, the headline (hypothesis) in **Table B.11** is true/false.

Table B.11. *Difference in fatty acid composition of pure and fortified sunflower oil (%w/w, on an oil basis) ± standard deviation (n=2) are represented in t-values. The headlines represent the hypothesis being accepted or rejected depending on if the t-value is significantly positive or negative. The level of significance is color coded according to the right hand side of the table*

Fatty acid	**Pure sunflower oil higher than *Porphyra***	**Pure sunflower oil higher than *Ulva***	**Fatty acid name**
C14:0	0.002	0.281	
C15:0	0.054	0.314	
C16:0	-0.630	-0.696	
C16:1	-0.667	0.774	
C17:1 n7	-7.607	-7.070	
C18:0	-0.692	-0.151	
C18:1 n9	-0.122	-0.801	
C18:1 n7	0.579	-0.109	
C18:2 n6	**0.072**	**-0.783**	**LA**
C18:3 n3	**-0.002**	-3.789	**ALA**
C20:0	-1.294	0.059	
C20:1 n9	-0.814	0.477	
C22:0	-1.398	0.627	
C24:0	-0.366	0.524	
Total	-0.033	-0.754	

Significance (v=2)		
$\alpha = 0.05$ t = 2.92	$\alpha = 0.01$ t = 6.965	$\alpha = 0.001$ t = 23.326

APPENDIX C

FORTIFICATION OF FISH OIL

STORAGE STUDY

PICTURES OF STORAGE STUDY

Pictures were taken at weekly interval as can be seen in **Figure C.1-C.7**.

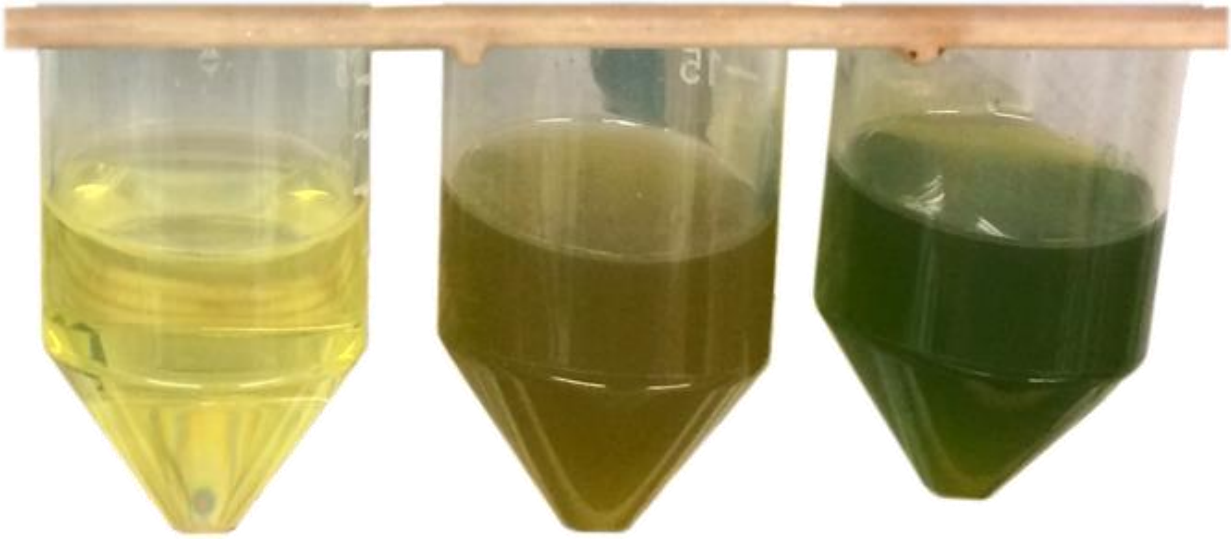


Figure C.1. *Initial picture of pure fish oil and fortified with Porphyra and Ulva*

Pure fish oil | Fortified with *Porphyra* | Fortified with *Ulva*

Figure C.2. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 7 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

Pure fish oil | Fortified with *Porphyra* | Fortified with *Ulva*

Figure C.3. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 14 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

Pure fish oil Fortified with *Porphyra* Fortified with *Ulva*

Figure C.4. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 21 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

Pure fish oil Fortified with *Porphyra* Fortified with *Ulva*

Figure C.5. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 28 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

Pure fish oil Fortified with *Porphyra* Fortified with *Ulva*

Figure C.6. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 35 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

Pure fish oil Fortified with *Porphyra* Fortified with *Ulva*

Figure C.7. *Picture of pure fish oil and fish oil fortified with Porphyra and Ulva after 42 days of daylight storage at room temperature (left), dark storage in room temperature (middle) and dark storage in fridge (right)*

FATTY ACID COMPOSITION OF PURE AND FORTIFIED FISH OIL

A two sample t-test assuming equal variance was preformed to compare the difference of each fatty acid between pure fish oil against fish oil fortified with *Porphyra* and *Ulva*. The level of significance is color coded according to **Table C.1**. Statistical data comparing the fatty acid profile in **Table 10** and **11** can be seen in **Table C.2 (A-C)** respectively. If the t-value is significant ($\alpha \leqslant 0.05$) and positive/negative, the headline (hypothesis) in **Table C.2 (A-C)** is true/false.

Table C.1. *T-values when comparing two triplicates assuming equal variance*

Significance (ν=4)		
$\alpha = 0.05$	$\alpha = 0.01$	$\alpha = 0.001$
t = 2.132	t = 3.747	t = 7.173

Table C.2. *Difference in fatty acid composition of pure and fortified fish oil (%w/w, on an oil basis) ± standard deviation (n=3) after (A) 0 days of storage, (B) 28 days of storage and (C) decrease of fatty acids during 28 days of storage. Values are represented in t-values*

	A		*B*		*C*		
Fatty acid	**Pure fish oil higher than *Porphyra***	**Pure fish oil higher than *Ulva***	**Pure fish oil higher than *Porphyra***	**Pure fish oil higher than *Ulva***	**Pure fish oil higher than *Porphyra***	**Pure fish oil higher than *Ulva***	**Fatty acid name**
<C18:0	3.957	1.420	-1.185	-0.726	2.092	0.949	
C18:0	1.320	1.547	-0.757	-0.021	1.453	0.699	
C18:1	3.276	-0.804	0.385	-0.223	1.011	-0.180	
C18:2 n6	**-0.271**	**-1.137**	**-1.166**	**-1.478**	**1.078**	**0.164**	**LA**
C18:3 n6	-1.958	-1.426	-0.930	2.153	-0.406	-1.996	
C18:3 n3	**1.217**	**-0.692**	**-1.383**	**-2.230**	**0.415**	**0.253**	**ALA**
C18:4 n3	1.201	2.105	-1.547	-2.434	0.568	0.690	
C20:1	1.873	0.349	0.727	0.378	0.101	-0.043	
C20:2 n6	2.445	4.914	-1.520	-1.308	3.923	2.438	
C20:3 n3	-0.575	1.158	-0.541	4.676	0.352	-2.058	
C20:4 n6	**-0.804**	7.279	**-0.918**	**-1.049**	**0.943**	**4.263**	**AA**
C20:4 n3	0.118	9.076	-0.531	1.502	1.012	4.452	
C20:5 n3	**1.938**	**1.567**	**-1.647**	**-2.253**	**2.548**	**2.200**	**EPA**
C21:5 n3	-0.678	1.590	-1.400	-2.240	1.939	3.303	
C22:1	1.335	1.106	-1.025	-1.609	3.613	2.566	
C22:5 n6	-0.439	3.453	-1.596	0.749	0.772	0.677	
C22:5 n3	-0.189	0.680	-0.731	-0.580	0.818	0.695	
C22:6 n3	**1.623**	**1.156**	**-2.023**	**-2.962**	**3.635**	**3.580**	**DHA**
C24:1 n9	0.669	0.427	-0.561	-0.753	0.658	1.192	
Total	3.397	1.174	-0.994	-1.160	2.042	1.170	**Total**

SENSORY ANALYSIS OF RANCID ODOR

The result from the sensory analysis for the pure and fortified fish oils stored in daylight at room temperature, in dark at room temperature and dark at 8˚C is shown in **Figure C.8 (A-C)**. No significant (α=0.05) differences were detected.

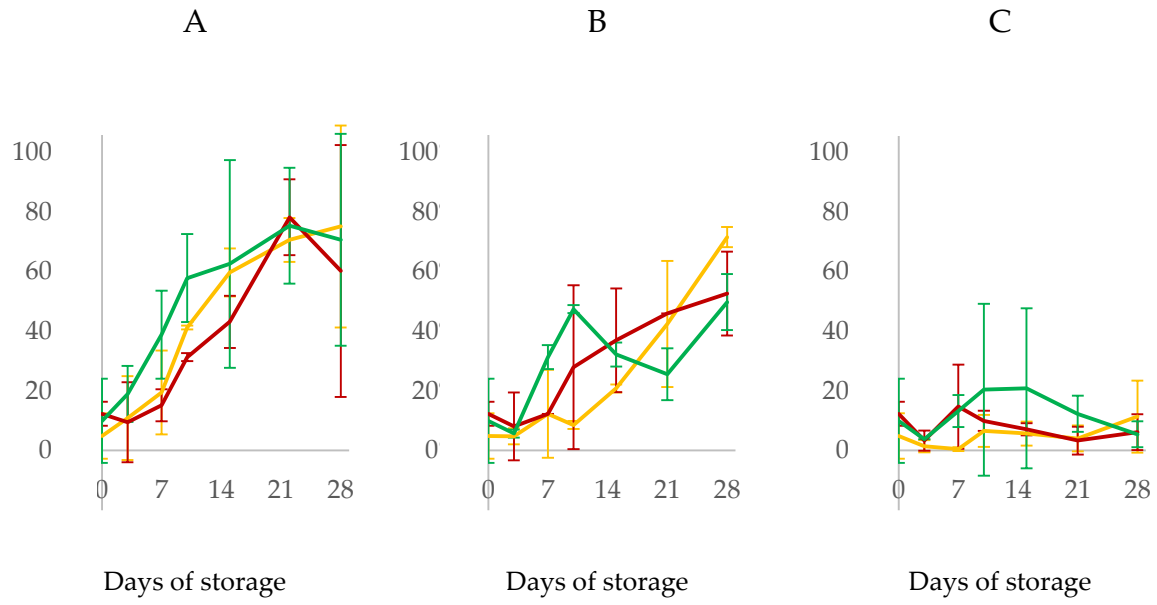


Figure C.8. *Intensity of rancid odor comparing pure fish oil (yellow) and fish oil fortified with Porphyra (red) and Ulva (green) stored in (A) daylight at room temperature, (B) dark at room temperature and (C) dark at 8˚C for 0-28 days (n=2)*

APPENDIX D

FORTIFICATION OF FISH OIL

IN VITRO DIGESTION

ASSAYS FOR ENZYMATIC ACTIVITY AND BILE SALT CONCENTRATION

α-AMYLASE

The very first step of the digestion takes place in the mouth where saliva hydrolyze starches into sugars. One of the most important component in human saliva which catalyze this reaction is *α*-amylase [64]. The enzyme is also found in pancreatic secretions, and due to high homology between porcine pancreatic *α*-amylase and human salivary *α*-amylase, the prior will be used in this study to simulate human saliva [65]. The activity of *α-amylase from porcine pancreas* (A3176, *Sigma-Aldrich*) was determined using a spectrophotometric method according to supplementary information in the Infogest-protocol [50]. A sodium phosphate buffer was prepared by dissolving 0.13g sodium phosphate (S-0751, *Sigma-Aldrich*), 0.28g sodium phosphate dibasic (S-5136, *Sigma-Aldrich*) and 0.039g NaCl in 100ml mQ-H_2O with a final pH adjusted to 6.9. A starch solution was prepared by mixing 0.25g *Starch from potato* (S2630, *Sigma-Aldrich*) with 20ml of the sodium phosphate buffer in a beaker covered with a loose lid. The beaker was put in a 95˚C shaking water bath for 15 minutes, whereafter it was cooled down to room temperature where mQ-H_2O was added to reach a final volume of 25ml. A color reagent was prepared by slowly dissolving 2.18g 3,5-dinitrosalicylic acid (DNSA) (128848, *Sigma-Aldrich*) in a flask with 80ml of 0.5M NaOH, which was heated to 70˚C on a magnetic stirrer with heating plate. When the DNSA was completely dissolved, 30g of potassium sodium tartrate (217255, *Sigma-Aldrich*) was added. When the potassium sodium tartrate was dissolved, the flask was let to cool down in room temperature before mQ-H_2O was added to a final volume of 100ml. Finally the enzyme was diluted to concentrations of 33.36 and 66.72μg/ml to get approximately 1 and 2 U/ml according to the activity on the package. 1ml of starch solution was pipetted into 6 different glass tubes and incubated in room temperature for 10 minutes. 1ml of each enzyme concentrations were pipetted in duplicates in separate glass tubes. The tubes were incubated for exactly 3 minutes before 1ml of color reagent was added whereafter the tubes were put in boiling water for exactly 15 minutes before put in an ice bath to cool. 9ml of mQ-H_2O was added to the tubes, whereafter they were mixed by inversion. The absorbance was measured at 540nm in a 10mm Quartz cuvette. As a blank for each enzyme concentration, the enzyme solution was put in the test tube while it was still in the boiling water bath.

As an external standard, maltose was mixed in mQ-H_2O to concentrations 0, 50, 200, 400, 600, 800, 1000 and 2000 μg/ml. 1ml of the maltose solution was mixed with 1ml of color reagent before put in boiling water bath for 15 minutes. 9ml of mQ-H_2O was added before the absorbance was measured at 540nm. The absorbance of the external standard was plotted against the concentration of maltose to create a standard curve

shown in **Figure D.1**. The trend line was forced through the origin, giving an R^2 equal to 0.9982 and an equation where Y = 0.6595X. The activity of α-amylase was then calculated according to **Equation D.1**. ΔA_{540} Sample is the absorbance of the sample subtracted with its respective blank. K is the slope from the standard curve. X is the weight [mg] of α-amylase used.

Equation D.1 $$U/mg = \frac{\Delta A_{540}\text{Sample}}{k * x}$$

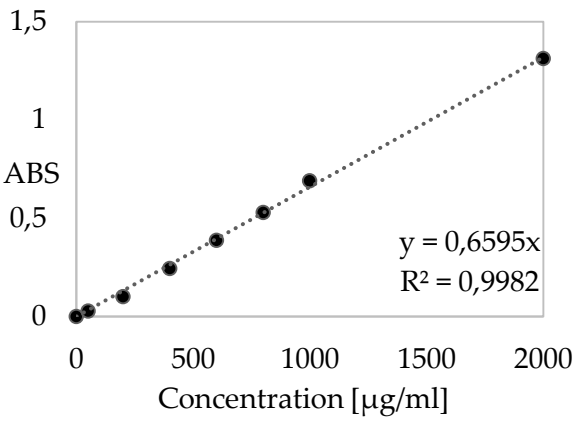


Figure D.1. *Standard curve for maltose in the range 0-2mg/ml recorded at 540nm*

GASTRIC LIPASE

When entering the gastric tract, the food is exposed to low pH and enzymes digesting fats and proteins. It is newly recognized that low amounts of lipase are secreted in the stomach. The use of gastric lipase is not recommended in the Infogest-protocol due to that gastric lipases similar to the ones found in humans were not commercially available at the time of publication of the protocol. However, since the focus of this study is to simulate the digestion of fat, lipase originating from *Rhizopus oryzae* was used. The enzyme have a different affinity compared to human lipase, giving rise to a different lipolysis [51]. This difference will be taken into account when deciding the concentration of this enzyme in part 3.3.4. The activity of *Lipase from Rhizopus oryzae* (80612, *Sigma-Aldrich*), used to simulate gastric lipase, was determined using the pH-stat technique with tributyrin (*SAFC, Sigma-Aldrich*) as a substrate. The gastric assay solution was prepared in a 50ml centrifuge tube with 1mg/ml sodium taurodeoxycholate (T0875, *Sigma-Aldrich*), 9mg/ml NaCl, 100µg/ml *Albumin, from bovine serum* (A3059,*Sigma-Aldrich*) in mQ-H_2O. 14.5ml assay solution was mixed with 0.5ml tributyrin in a 30ml vial (*Cerbo laboratory vial 36*, *Nolato*) and heated to 37˚C in a *Julabo SW23* shaking water bath. A 20mm magnetic bar was put in the vial which was then put in a beaker with 37˚C ambient water on a *IKA® yellowline MSH basic* magnetic stirrer with heating plate. The solution was titrated to pH8 by using 0.1M NaOH (*Scharlau*) in a *907 Titrando* titrimeter. 1mg/ml lipase from *Rhizopus oryzae* in mQ-H_2O was prepared in a 1.5ml *Microcentrifuge Tube*, whereafter 50µl was pipetted into the mixture. The titrimeter kept the pH constant, at pH8, and the volume of NaOH titrated was recorded by using the software *tiamo™ version 2.4*. The activity was measured in tributyrin units (TBU)/mg, where 1 TBU is the amount of enzyme that can release 1 µmol of butyric acid from the triglyceride butyrin per minute at 37˚C and pH 8. This is equivalent with µmol NaOH titrated/minute at 37˚C and pH 8.

PEPSIN

To simulate the digestion of proteins, *Pepsin from porcine gastric mucosa* (P7000, *Sigma-Aldrich*) was be used. The activity was determined using a spectrophotometric method originally developed by *Anson* (1938), with *Hemoglobin from bovine blood* (51290, *Fluka, Sigma-Aldrich*) as a substrate [66]. The substrate was prepared by dissolving 0.5g hemoglobin powder with mQ-H_2O to a final volume of 25ml, with pH adjusted to 2 by the addition of 300mM HCl. A stock solution of 1mg/ml pepsin in 150mM NaCl (pH6.5) was mixed and diluted to a 0.1mg/ml working solution. The working solution was diluted with 10mM HCl to enzyme sample concentrations of 5, 10, 15, 20, 25, 30, 35 µg/ml. A half ml of hemoglobin was pipetted into 14 empty 13ml centrifuge tubes and incubated in a 37˚C water bath until samples had reached ambient temperature. 100µl of enzyme sample in the aforementioned concentrations was added to 7 of the tubes which was then left to incubate for exactly 10 minutes. 1ml of 5% trichloroacetic acid (TCA) (*Sigma-Aldrich*) solution in mQ-H_2O was added to the 14 tubes. 100µl of each enzyme sample concentration was added to the tubes which had yet not received any enzymes. These tubes were to work as a blank for each concentration. All samples were centrifuged for 30 minutes at 6000g. The spectrophotometer was set to 280nm and blanked with 5% TCA before the absorbance of each clear supernatant was measured. The activity was calculated by using **Equation D.2** derived from *Anson* (1938) [66]:

Equation D.2

$$\mathrm{U/mg} = \frac{(\mathrm{A_{280}Sample} - \mathrm{A_{280}Blank})}{0.001 * t * X}$$

(A_{280} Sample - A_{280} Blank) is the difference in absorbance, different for each enzyme concentration. The increase in absorbance 1U will produce at pH2 and 37˚C is 0.001, according to the Anson unit definition. T is the incubation time in minutes, equal to 10 minutes. X is the enzyme sample concentrations in mg/ml.

PANCREATIC LIPASE

The enzymes occupying our intestinal tract was simulated with the use of *Pancreatin from porcine pancreas* (P1750, *Sigma-Aldrich*). The activity of lipase was determined using the same pH-stat technique as described in previous part. In contrast, the pancreatic assay solution contained 36µg/ml tris(hydroxymethyl)aminomethane (*Sigma-Aldrich*), 9mg/ml NaCl, 200µg/ml $CaCl_2 \cdot 2H_2O$ (*Scharlau*) and 2.08mg/ml sodium taurodeoxycholate in mQ-H_2O. The pH was kept stable at 6 instead of 8, and pancreatin from porcine pancreas was used instead of lipase from *Rhizopus oryzae*.

PANCREATIC TRYPSIN

The activity of trypsin in *Pancreatin from porcine pancreas* was determined using a spectrophotometric method with N_{α}-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) (T4626, *Sigma-Aldrich*) as a substrate. A buffer was prepared with 5.57mg/ml tris(hydroxymethyl)aminomethane and 1.7mg/ml $CaCl_2 \cdot 2H_2O$ in mQ-H_2O with pH adjusted to 8.1 by using 1M HCl (*Sigma-Aldrich*). The spectrophotometer was blanked with 1.3ml buffer, 150µl 10mM TAME and 50µl 1mM HCl and tuned to measure absorbance at 247nm every 6 seconds for 10 minutes. The samples contained 1.3ml buffer, 150µl 10mM TAME and 50µl of 1 and 0.5mg/ml pancreatin in 1mM HCl. The enzymatic kinetics was measured by using *Cary WinUV Kinetics Application* software. The activity was calculated by using **Equation D.3** derived from *B. C. Hummel* (1959) [67]:

Equation D.3

$$\mathrm{U/mg} = \frac{(\Delta A_{247}\mathrm{Sample} - \Delta A_{247}\mathrm{Blank}) * \mathrm{V}}{\varepsilon * m_{trypsin}}$$

(ΔA_{247} Sample - ΔA_{247} Blank) is the linear slope when the sample absorbance is subtracted with the blank absorbance and plotted against the time. V is the reaction volume in µl, equal to 1500µl. The molar extinction coefficient ε for the change in absorbance at 247nm with cleavage of TAME is 540 [68]. This is used since one unit is defined as the hydrolysis of 1 µmol TAME per minute at pH8.1 and 25˚C.

APPENDIX E

FORTIFICATION OF FISH OIL

STORAGE STUDY II

Pure fish oil and fish oils fortified with the Swedish brown seaweed species *Saccharina latissima* and *Fucus vesiculosus* were evaluated based on the aldehydes production, and a sensory analysis of rancid odor over storage. See **Figure E.1** for a picture of pure fish oil and fish oil fortified with *Saccharina* and *Fucus.* Even though both species are categorized as brown seaweed, the fish oil fortified with *Fucus* showed a dark green color.

Figure E.1. *Initial picture of pure fish oil and fortified with Saccharina* and *Fucus*

ALDEHYDES

See **Figure E.2** for a diagrams of the aldehydes MDA, HHE and HNE over daylight storage at room temperature for 21 days. The amount of MDA was significantly higher in the pure fish oil after 7 and 21 days of storage, compared to both fortified fish oils. The amount of HHE and HNE on the other hand was significantly higher in both the fortified oils, compared to the pure fish oil, after 7 and 21 days of storage.

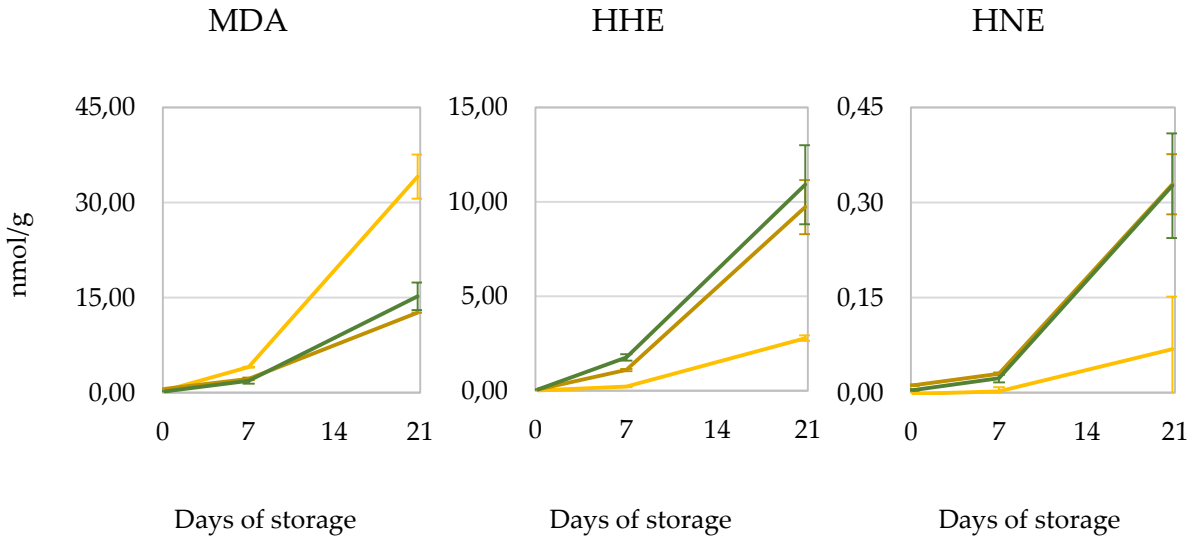


Figure E.2. *Concentration of aldehydes (MDA, HHE and HNE) in the pure fish oil (yellow) and fish oils fortified with Saccharina latissima (brown) and Fucus vesiculosus (green) at day of fortification, after 7 days and after 21 days of daylight storage at room temperature ± standard deviation (n=2)*

SENSORY ANALYSIS OF RANCID ODOR

The sensory analysis of rancid odor for the pure fish oil and fish oil fortified with *Saccharina* and *Fucus* can be seen in **Figure E.3**. No significant differences (α=0.05) were found.

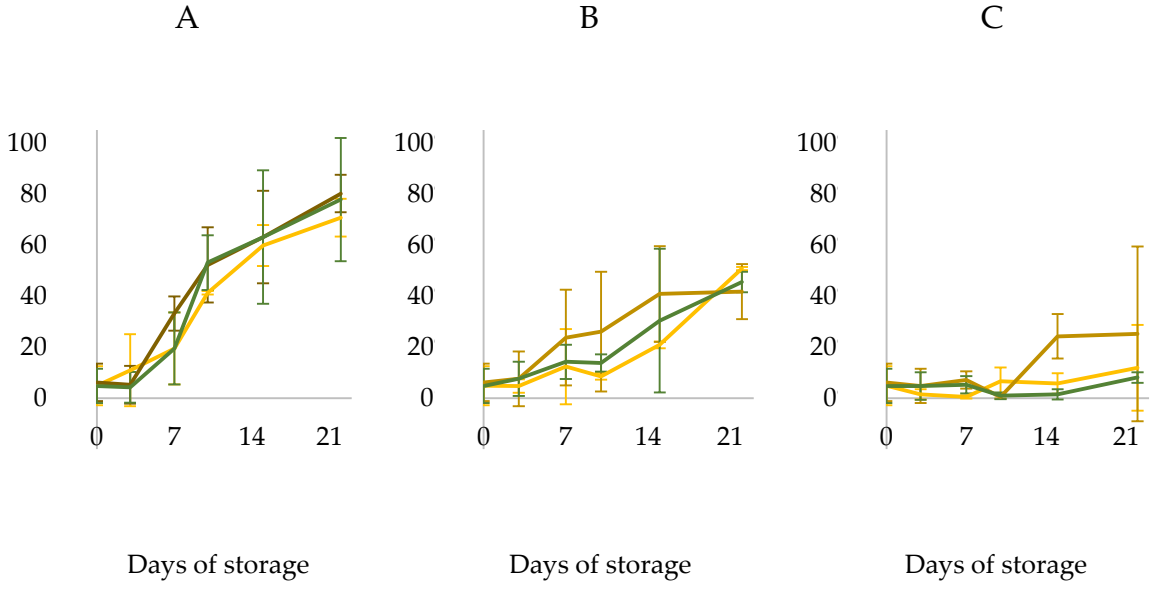


Figure E.3. *Intensity of rancid odor comparing pure fish oil (yellow) and fish oil fortified with Saccharina (brown) and Fucus (dark green) stored in (A) daylight at room temperature, (B) dark at room temperature and (C) dark at 8˚C for 0-22 days (n=2)*