Extraction, characterization and application of antioxidants from the Nordic brown alga *Fucus vesiculosus*

PhD thesis by Ditte Baun Hermund

National Food Institute
Technical University of Denmark

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DATA SHEET

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                  the Nordic brown alga *Fucus vesiculosus*

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PREFACE

This PhD project entitled "Extraction, characterization and application of antioxidants from the Nordic brown alga *Fucus vesiculosus*" was carried out at the National Food Institute (DTU Food), Technical University of Denmark (DTU). The work was supervised by Professor Charlotte Jacobsen (main supervisor) from DTU Food and Associate Professor Kristian Fog Nielsen (co-supervisor) from DTU Systems Biology. The project started in May 2012 and continued until February 2016, interrupted for 8 months due to maternity leave.

The aim of this project was to extract and characterise highly antioxidative polyphenolic secondary metabolites, phlorotannins, derived from Nordic brown alga *Fucus vesiculosus*. Furthermore, it was to study possible applications for *F. vesiculosus* extracts rich in phlorotannins to inhibit lipid oxidation in food and skin care products.

The PhD work was part of the project "Novel bioactive seaweed based ingredients and products" which was part of the Nordic marine 3 innovation programme financed by Nordic Innovation. Our part in this project was to study the application possibilities of extracts from Icelandic *F. vesiculosus* plants. This was done in collaboration with Matís in Iceland. An additional part of the PhD project, was to extract, identify and characterise phlorotannins in Nordic *F. vesiculosus* plants. Integrated during this part was a research stay at Kemicentrum at Lund University in Sweden.

I was awarded a travel grant from the European Section of AOCS to participate in the 105th AOCS Annual Meeting & Expo in San Antionio, Texas, USA, May 4-7, 2014. Furthermore, I was granted the Young Scientist Award from Nordic Lipid Forum which supported my participation in the 28th Nordic Lipid Symposium in Reykjavik, Iceland, June 3-6, 2015. The stay in Iceland also gave me a possibility to visit Matís and their research facilities.

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SUMMARY

Marine algae are a huge underutilized resource in the Nordic countries with a potential to be used in the development of new natural ingredients for the food, cosmetics and pharmaceutical industry. Such ingredients can act as natural preservatives and prevent product deterioration during storage, in particular in the form of rancidity due to oxidation of unsaturated fatty acids in the products.

A characteristic feature of *Fucus vesiculosus*, also known as bladder wrack, is a high content of phlorotannins – a particular type of polyphenol group. Previous studies have shown positive correlations between the phlorotannin content and radical scavenging capacity of extracts derived from Nordic *F. vesiculosus*. Radical scavenging capacity is an important antioxidant property in terms of preventing the oxidation of unsaturated fatty acids. The high content of antioxidative phlorotannins in *F. vesiculosus* therefore makes this alga particularly attractive for the development of new natural antioxidants. While the *in vitro* antioxidant properties of *F. vesiculosus* extracts are widely studied, studies evaluating the antioxidant efficacy of such extracts in food and skin care products are scarce.

This PhD study investigated the possibilities of using extracts from Nordic *F. vesiculosus* as natural antioxidants in food and skin care products. All tested food products were fortified with fish oil rich in polyunsaturated omega-3 fatty acids. The fish oil was added specifically in order to examine the effectiveness of the antioxidants in systems which are more likely to oxidize compared with conventional products. The products tested were all oil-in-water emulsions except for granola bars, which were instead added 70% fish oil-in-water emulsions. Tests were made on a selections of extracts made from water, acetone, and ethanol, as well as a fraction of purified phlorotannins. Investigations also highlighted the influence of the extraction medium on the antioxidant properties, the phlorotannin content as well as other co-extracted substances. Moreover, it was examined which phlorotannins were present in each of the extracts, and how each specific phlorotannin contributed to the overall antioxidant activity.

All extracts examined and also the phlorotannin-rich fraction were somewhat able to improve the oxidative stability of the food and skin care products. The effectiveness of these extracts was to a large degree dependant on their antioxidant properties and composition, which in turn depended on the extraction medium used. In general, water was efficient in extracting iron chelating compounds. However, it was also found that water was not effective in extracting phlorotannins, and that the iron chelating ability, according to our results, to a greater extent was due to the presence of the pigment 19-hex-fucoxanthin. It has also been discussed whether algal sugars with iron chelating ability may be extracted with water and hence affect the antioxidant properties of the

water extract. However, this aspect was not investigated. The high iron chelating ability of the water extract proved particularly effective in FO-enriched mayonnaise. Previous studies have also shown that iron chelating ability is an important property of antioxidants to work efficiently in this particular food. Acetone and ethanol were highly effective in extracting phlorotannins, which were found to have good radical scavenging capacity as well as reducing power. In addition, these phlorotannins exhibited a high affinity to the interface between the hydrophilic and the hydrophobic phase, compared to phlorotannins extracted with water. The more amphiphilic phlorotannins were also found to be effective antioxidants in FO-enriched granola bars. It was examined from microscopy how the emulsified fish oil added seaweed extracts localized when added to the granola bars. Emulsions added extracts with more amphiphilic phlorotannins clearly improved incorporation of the fish oil emulsions into the granola bars, which in turn had a major impact on the oxidative stability of these products. It was concluded that the surface active phlorotannins were important radical scavengers in granola bars. These phlorotannins are chain-breaking antioxidants that deactivate lipid radicals formed in the first part of lipid oxidation. In addition, it was discussed whether some of these phlorotannins also regenerated antioxidative tocopherols from the oil phase.

A structural characterization and on-line detection of phlorotannins in the purified fraction was carried out in support of a further characterisation of phlorotannins and how they each contribute to the overall antioxidant activity. By mass spectrometry 13 phlorotannin isomers were identified with molecular weights between 374 and 870 Da (3 to 7 phloroglucinol units). It was found that the antioxidant activity is decreasing with increased molecular weight and hence with increased polymerization of the phlorotannins.

This PhD work has contributed basic knowledge of relevance to future large scale development of natural antioxidants from seaweeds to the benefit of the food, cosmetic and pharmaceutical industrial sectors. It is clearly demonstrated that it is possible to produce antioxidants from seaweed thallus, and also that it is possible to use alternative environment-friendly extraction methods. In addition, the studies highlight examples of application possibilities of seaweed extracts as natural antioxidants, e.g. in the formulation of functional foods enriched with fish oil.

RESUMÉ

Tang er en stor uudnyttet ressource i Norden, som har potentiale til at kunne anvendes til udvikling af nye naturlige ingredienser til fødevare-, kosmetik- of lægemiddelindustrien. Sådanne ingredienser kan virke som naturlige konserveringsmidler for at undgå produktforringelse under lagring, bl.a. i form af harskning grundet oxidation af umættede fedtsyrer i produkterne.

Fucus vesiculosus, også kaldet blæretang, er særligt kendetegnet ved at have et højt indhold af en særlig slags polyphenoler, kaldet phlorotanniner. Tidligere studier har påvist en sammenhæng mellem et højt phlorotanninindhold og høj *in vitro* antioxidant aktivitet, særligt radikal indfangningsevne, af ekstrakter fra bl.a. nordisk *F. vesiculosus.* Radikalindfangningsevnen er en vigtig egenskab i forhold til at forhindre oxidation af umættede fedtsyrer. Det høje indhold af de antioxidative phlorotanniner i *F. vesiculosus* gør derfor denne art særlig attraktiv til udvikling af nye naturlige antioxidanter udvundet fra tang.

Mens studier, der undersøger *in vitro* antioxidant egenskaber i antioxidant assays af *F. vesiculosus* ekstrakter, er udbredte, er der noget længere mellem studier, som behandler antioxidant-aktiviteten af lignende ekstrakter i bl.a. fødevare- og hudplejeprodukter.

Dette ph.d. studie undersøgte mulighederne for at anvende ekstrakter fra nordisk *F. vesiculosus* som naturlige antioxidanter i fødevare- og hudplejeprodukter. Alle testede fødevareprodukter var berigede med fiskeolie rig på flerumættede omega-3 fedtsyrer. Fiskeolien blev tilsat netop for at undersøge, hvor effektive antioxidanterne var i systemer, som er mere tilbøjelige til at oxidere sammenlignet med konventionelle produkter. De testede produkter var alle olie-i-vand emulsioner på nær müslibarerne, som i stedet var tilsat 70% fiskeolie-i-vand emulsioner. Et udvalg af ekstrakter lavet på vand, acetone og ethanol, samt en fraktion af oprensede phlorotanniner blev testet. Indflydelsen af ekstraktions-mediet på de antioxidative egenskaber, indholdet af phlorotanniner og de øvrige ekstraherede stoffer blev kortlagt. Det blev ydermere undersøgt, hvilke phlorotanniner der er til stede i disse ekstrakter, og hvordan hver enkel phlorotannin kan bidrage til den overordnede antioxidant-aktivitet.

Alle undersøgte ekstrakter og den phlorotannin-rige fraktion var i nogen grad i stand til at forbedre den oxidative stabilitet af fødevare- og hudplejeprodukter. Effektiviteten af disse ekstrakter afhang dog i høj grad af deres antioxidative egenskaber og komposition, som igen afhang af, hvilket ekstraktionsmedie der var blevet brugt under ekstraktionen. Generelt var vand effektivt til at ekstrahere stoffer med jernkelerende evne. Dog viste det sig, at vand ikke var effektivt til at ekstrahere phlorotanniner, og at den jernkelerende evne ifølge vores resultater i højere grad skyldes tilstedeværelsen af 19-hex-fucoxanthin. Dog blev det også diskuteret, hvorvidt sukre med

jernkelerende evne muligvis i højere grad ekstraheres med vand og dermed kan have indflydelse på vandekstraktets antioxidative egenskaber. Dette blev dog ikke undersøgt nærmere. Den høje jernkelerende evne for vandekstraktet viste sig særligt effektiv i fiskeolieberiget mayonnaise. Tidligere studier har også vist, at jernkelerende aktivitet er en vigtig egenskab hos antioxidanter, som er effektive i netop denne fødevare. Acetone og ethanol var i høj grad effektive til at ekstrahere phlorotanniner, som viste sig at have gode radikalindfangnings- samt reducerende evner. Ydermere udviste disse phlorotanniner en høj affinitet til grænsefladen mellem den hydrofile og hydrofobe fase sammenlignet med de phlorotanniner, som var ekstraheret med vand. De mere amphiphile phlorotanniner viste sig også at være effektive antioxidanter i fiskeolieberigede müslibarer. Med mikroskopi blev det undersøgt, hvordan for-emulgerede fiskeolieemulsioner tilsat tangekstrakter lokaliseredes, når de kom i müslibardejen. Det viste sig, at ved tilsætningen af emulsioner, hvor de mere amphiphile phlorotanniner var til stede, forblev oliedråberne i højere grad intakte, hvilket havde stor betydning for den oxidative stabilitet af müslibarerne. Det blev konkluderet at de overfladeaktive phlorotanniner var vigtige radikalindfangere i müslibarer. Disse phlorotanniner virkede i høj grad som kædebrydende antioxidanter ved at deaktivere lipidradikaler dannet i første del af lipidoxidationen. Derudover blev det diskuteret, hvorvidt nogle af disse phlorotanniner også regenererede antioxidative tocoferoler i oliefasen.

Ved nærmere undersøgelse af phlortanninernes sammensætning, og hvordan disse hver især bidrog til den overordnede antioxidant aktivitet, blev der foretaget strukturkarakterisering samt on-line detektion af phlorotanninerne i den oprensede fraktion. Ved massespektrometri blev der identificeret 13 phlorotanninisomerer med molekylevægte mellem 374 og 870 Da (3 til 7 phloroglucinolenheder). Det viste sig, at antioxidant aktiviteten var faldende med øget molekylevægt og dermed også med øget polymerisering af phlorotanninerne.

Dette ph.d. arbejde har bidraget med central basisviden, som er nødvendig for en fremtidig udvikling af naturlige antioxidanter udvundet fra tang i industriel skala til gavn for fødevare-, kosmetik- og lægemiddelindustrien. Studierne har således demonstreret, at det er muligt at producere antioxidative tangekstrakter og har også åbnet mulighederne for at bruge alternative miljøvenlige ekstraktionsmetoder til ekstraktion af antioxidanter fra tang. Derudover har studierne anvist eksempler på anvendelsesmuligheder der er for sådanne tangekstrakter som naturlige antioxidanter bl.a. i formuleringen af funktionelle fødevarer beriget med fiskeolie.

LIST OF PUBLICATIONS

Publications

Paper I	**Hermund, D.B.**, Yeşiltaş, B., Honold, P., Jónsdóttir, R., Kristinsson, H.G., & Jacobsen, C. (2015). Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts in vitro and in fish-oil-enriched milk and mayonnaise. *Journal of Functional Foods*, *19,* 828-841.
Paper II	Honold, P.J., Jacobsen, C., Jónsdóttir, R., Kristinsson, H.G., & **Hermund, D.B.** (2015). Potential seaweed based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise. *European Food Research and Technology*, DOI 10.1007/s00217-015-2567-y.
Paper III	Karadağ, A.*, **Hermund, D.B.***, Søgaard Jensen, L.H., Andersen, U., Jónsdóttir, R., Kristinsson, H.G., Alasalvar, C., & Jacobsen, C. (2015). The effect of novel brown seaweed based antioxidants on the oxidative stability and microstructure of 5% fish-oil-enriched granola bars. *European Journal of Lipid Science and Technology, Submitted* (Dec 2015) (*joint authorship).
Paper IV	**Hermund, D.B.***, Karadağ, A.*, Andersen, U., Jónsdóttir, R., Kristinsson, H.G., Alasalvar, C., & Jacobsen, C. (2016). Oxidative Stability of Granola Bars Enriched with Multilayered Fish Oil Emulsion in the presence of Novel Brown Seaweed Based Antioxidants. Journal of Agricultural and Food Chemistry, *draft* (*joint authorship).
Paper V	Poyato, C., Raagaard Thomsen, B., **Hermund, D.B.**, Astiasarán, I., Jónsdóttir, R., Kristinsson, H.G., & Jacobsen, C. (2016). Antioxidant effect of aqueous and acetonic extracts of *Fucus vesiculosus* on oxidative stability of cosmetic emulsions. European Journal of Lipid Science and Technology, *Submitted* (Feb 2016).
Paper VI	**Hermund, D.B.,** Plaza, M., Turner, C., Jacobsen, C., & Nielsen, K.F. (2016). Structure dependent antioxidant capacity of phlorotannins from Icelandic *Fucus vesiculosus* by UHPLC-DAD-ECD-QTOFMS (*Short communication*). Food Research International*, Submitted (March 2016).*

CONFERENCE CONTRIBUTIONS

Conference contributions

Oral presentations	"Novel bioactive algae based food ingredients", Alga Symposium, Lyngby, Denmark, February 2014.
	"Novel bioactive algae based food ingredients", 105th AOCS Annual Meeting & Expo, AOCS European Section, Travel grant receiver, San Antonio, Texas, May 2014.
	"Bioactive compounds extracted from seaweed and application in food systems", ICAB, Lyngby, Denmark, August 2014.
	"Nordic Seaweed extracts as natural antioxidants in omega-3 PUFA enriched granola bars", Young Scientist Award, 28th Nordic Lipidforum Symposium, Reykjavik, Iceland, June 2015.
	"Seaweed based antioxidants – analysis and application", Nordic Seaweed Conference, Grenaa, Denmark, October 2015.
	"Polyphenolic compounds from *Fucus vesiculosus* and their antioxidant activity", International Seaweed Symposium, Lyngby, Denmark, June 2016 (abstract accepted).
Posters	"Seaweed based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise" by DB Hermund, P Honold, R Jónsdóttir, HG Kristinsson, and C Jacobsen, 12th Euro Fed Lipid Conference, Montpellier, France, September 2014.
	"The use of natural antioxidants in omega-3 rich food products" by R Jónsdóttir, DB Hermund, C Jacobsen, and HG Kristinsson, 28th Nordic Lipidforum Symposium, Reykjavik, Iceland, June 2015.
	"Antioxidant activity and tentative identification of polyphenolic compounds from the Nordic Brown algae *Fucus vesiculosus*", 1st International Symposium on Lipid Oxidation and Antioxidants, Porto, Portugal, June, 2016.

ABBREVIATIONS

BHT	Butylated hydroxytoluene
CHI	Chitosan
CLSM	Confocal light scanning microscopy
DAD	Diode array detector
DHA	Docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
dw	Dry weight
EAF	LLP ethyl acetate fraction
ECD	Electrochemical detector
EDTA	Ethylenediamine tetraacetic acid
EIC	Extracted ion chromatogram
EE	SLE ethanol extract
EPA	Eicosapentaenoic acid
ESEM	Environmental scanning electron microscopy
ESI	Electrospray ionisation
F.	*Fucus*
FA	Fatty acid
FO	Fish oil
GAE	Gallic Acid Equivalent
GC	Gas chromatography
H	Hydrogen
HPLC	High performance liquid chromatography
L·	Lipid alkyl radical
LC	Liquid chromatography
LH	Unsaturated lipid
LLP	Liquid-liquid partitioning
LO·	Lipid alkoxyl radical
LOOH	Lipid hydro peroxide
M	Metal
MS	Mass Spectrometry

m/z	Mass to charge
Na-cas	Sodium Caseinate
NMR	Nuclear magnetic resonance
o/w	Oil-in-water
PCA	Principal component analysis
PLE	Pressurised liquid extraction
PUFA	Polyunsaturated fatty acids
PV	Peroxide value
qTOF	Quadrupole-time-of-flight
ROS	Reactive oxygen spices
SD	Standard deviation
SEM	Scanning electron microscopy
SLE	Solid-liquid extraction
Toc	Tocopherol
TPC	Total phenolic content
W	Water
WE	SLE water extract (whole seaweed)
WoE	SLE water extract (old part)
WyE	SLE water extract (young part)

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Chapter 1: Introduction

1.1. Background

In 2012, 23.8 million tons of fresh seaweed was harvested worldwide. Approximately 95 % was produced in aquaculture mainly in Asia, while the rest is harvested from the wild both in Asia and in Europe (Norway, France and Ireland) (FAO, 2014; Hansen, 2013). Seaweed has historically been used for human consumption, feed and fertilizer in the Nordic countries.

Seaweed is at present a highly underutilized resource in the Nordic countries, however, with great potential. At the moment, the utilization of seaweeds in the Nordic countries is restricted to inexpensive low-grade seaweed products or used for research. In Iceland there is thus only one company harvesting seaweeds at a sizable scale and processing seaweed meal for export. Hence there is no final processing of active ingredients from seaweeds and therefore minimal value addition to this material. Out of an estimated many millions of metric tons of seaweed biomass in Iceland, less than 20.000 tons of marine seaweed are harvested, representing a minute fraction of what could be harvested and utilized. The Nordic countries are in a unique position to create significant value from their very abundant seaweed resources.

1.1.1. The need for novel natural antioxidants

Lipid oxidation is recognized as a major problem in quality maintenance during storage of lipid rich products, especially in oxidation of long chain polyunsaturated fatty acids (PUFAs). Oxidative degradation of lipids to lipid hydroperoxide and further to volatile secondary oxidation products can result in repugnant flavours, nutrient loss and even in generation of toxic compounds endangering human health (Yagi, 1987).

To overcome this problem synthetic antioxidants are widely used by the industry to enhance oxidative stability of lipid rich products. However, restrictions in the use of synthetic antioxidants have been enforced because of their health risks and toxicity (Branen, 1975; Linderschmidt et al., 1986). Hence there is a significant interest in and demand for replacing synthetic antioxidants with natural plant-based alternatives, not only due to safety issues but also due to a generic interest in sustainable and natural solutions (Halliwell, 1996).

1.1.2. Potential antioxidant substances in seaweed

Seaweeds contain a wide range of bioactive substances like sulphated polysaccharides, peptides, amino acids, and polyphenols, all exhibiting antioxidant activity (Holdt & Kraan, 2011). Therefore, natural antioxidants derived from seaweeds have great potential for improving oxidative stability of

products with lipid oxidation challenges, and additionally in providing the product with functionalities like health promoting benefits or anti-aging effects.

During recent years a number of studies have reported that seaweed extracts demonstrated strong antioxidant properties due to the presence of secondary metabolites, i.e. *phlorotannins*, which are the dominant polyphenolic compounds in brown algae (Phaeophyceae) (Jiménez-Escrig et al., 2001; Wang et al., 2009; 2012).

It has been demonstrated that phlorotannins are potent metal chelators and free radical scavengers (Jiménez-Escrig et al., 2001; Ahn et al., 2007; Kim et al., 2009). Extracts from brown algal species from both Iceland and Denmark (*Fucus vesiculosus* L. and *Fucus serratus* L.) have high total phenolic content as determined by the *Folin–Ciocalteu assay* and also higher antioxidant activity in comparison with other algal species (red and green algal) which have a lower phenolic content (Wang et al., 2009; Farvin & Jacobsen, 2013). In *F. vesiculosus* and *F. serratus* collected from the west coast of Ireland a similar relationship between high antioxidant activity and high phenolic content was also evident (O'Sullivan et al., 2011).

1.1.3. Extraction and identification of phlorotannins

The correlation between high antioxidant activity and high phenolic content raise an interest in utilizing brown seaweeds for the development of novel natural antioxidants rich in phlorotannins.

Successful extraction of phlorotannins has been obtained using solvents such as acetone and ethanol, which also have been found to extract higher yields of phenolic compounds compared with water (Wang et al., 2009, 2012; Farvin & Jacobsen, 2013). Furthermore, simple purification of extracts obtained by solvent partitioning using ethyl acetate has been found to increase the phlorotannin content (Wang et al, 2012). However, traditional solvent extraction might for environmental reasons be an unwanted extraction method in a production of natural novel antioxidants.

Other extraction solutions such as pressurized liquid extraction (PLE), which is a more environmental friendly technique, has been found to successfully extract phenolic compounds from natural samples (Plaza et al., 2013). PLE is based on the use of a combination of temperature and pressure where solvents are maintained in their liquid state during the whole extraction process. Typically water is used exclusively as extractant or in combination with a co-solvent, i.e. ethanol. The advantages of PLE are a reduction in the amount of solvent used, and a shorter extraction time. The process is automated and retains the sample in an oxygen and light-free environment in contrast to traditional solvent extraction. This method could be an alternative way of extracting phlorotannins from brown algae and is yet to be tested.

Fucus vesiculosus contains a wide range of other bioactive compounds beside phlorotannins, such as pigments (carotenoids, chlorophylls), different tocopherols, sulphated polysaccharides (fucoidans), amino acids, metals and mono- and polyphenols (Holdt & Kraan, 2011; Farvin & Jacobsen, 2013). Some of these compounds are possibly co-extracted when targeting extraction of phlorotannins. The compounds can contribute both antioxidatively and prooxidatively to the functionality of *F. vesiculosus* extracts. Hence, identification of these bioactive co-extracted compounds is necessary in order to determine their role in seaweed extracts.

Due to the dominance of phlorotannins, the total phenolic content is often related to the content of phlorotannins (Wang et al., 2009; Farvin & Jacobsen, 2013; Tierney et al., 2013). Hence, whereas many studies determine the total phlorotannin content of brown algal tissue, and relate this to the antioxidant activity, only few studies have considered identification of individual phlorotannins (Ferreres et al., 2012; Tierney et al., 2013; Yotsu-Yamashita et al., 2013). Moreover, no studies have considered the antioxidant activity of individual phlorotannins extracted from *F. vesiculosus*, although one study found that fractions of Icelandic *F. vesiculosus* extracts, rich in Fucudiphloroethol E, a phlorotannin isomer, had a higher *in vitro* antioxidant activity indicating that this could be the phlorotannin with the highest antioxidant activity (Wang et al., 2012). A full identification and characterization of phlorotannins requires application of advanced analytical methods including mass spectrometry (MS).

1.1.4. *Antioxidant evaluation and the role of phlorotannins*

The antioxidant mechanism of seaweed extracts can be determined by *in vitro* studies. Free radical scavenging is the main mechanism by which antioxidants work by H-donation and termination of the oxidation process by converting free radicals into more stable products. Several methods have been developed to determine the antioxidant activity by assessing their scavenging of synthetic radicals such as 2,2-diphenyl-1-picrylhydrazyl (DPPH•) (Kurechi et al., 1980). Ferrous ion chelating is another antioxidant mechanism, which is important in food and skin care products, where antioxidants reduce oxidized intermediates into a more stable form. It is possible to determine the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} by *in vitro* antioxidant assays (Decker & Welch, 1990).

As mentioned a positive correlation between phlorotannin content and antioxidant activity of *F. vesiculosus* extracts have been demonstrated. However, identification and characterization of these highly antioxidative phlorotannins must be carried out to give a more clear indication of the role of phlorotannins in the overall antioxidant activity of *F. vesiculosus* extracts. Zettersten et al. (2009) developed a fast separation system for characterizing polyphenolic antioxidants in complex samples on-line using high-performance liquid chromatography with diode array, electrochemical, and mass spectrometry detection (HPLC-DAD-ECD-MS/MS). Development of a similar method for

phlorotannins would give us a tool to identify and determine the antioxidant activity of individual phlorotannins.

1.1.5. Application possibilities of F. vesiculosus extracts

The *in vitro* antioxidant properties are only an indicator for how antioxidants can act in real food or skin care products. To fully evaluate the antioxidant activity of extracts, they should also be evaluated in application. Moreover, the effects of antioxidants cannot be extrapolated from one product to another but must be evaluated in each case, as the efficacy of the same antioxidant is highly product dependent (Nielsen et al., 2004; Jacobsen et al., 2008).

Several foods and skin care products are oil-in-water (o/w) emulsions, such as milk, mayonnaise and facial cream. Studies on oxidative stability of fish-oil-enriched milk and mayonnaise added antioxidants have been performed (Alemán et al., 2015; Sørensen et al., 2015; Haahr & Jacobsen, 2008; Jacobsen et al., 2001). Granola bars or fitness bars enriched with fish-oil-in-water emulsion has also been studied (Nielsen & Jacobsen, 2009; Horn et al., 2009).

Fish oil (FO) is rich in n-3 long chain (LC) PUFAs, like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Products enriched with FO will, however, be more susceptible to lipid oxidation, leading to the development of undesirable off-flavours and reduced shelf life (Jacobsen et al., 2000; Let et al., 2003; Let, et al., 2007). Therefore, controlling lipid oxidation of FO-enriched foods is challenging but will give a good indication of how efficient seaweed based antioxidants can be.

The functionality of skin care products, like facial cream, is highly dependent on the presence of intact PUFAs. Lipid oxidation of fatty acids like linoleic acid will lead to development of undesirable odours and flavours and decreased effect of this fatty acid in the skin care product (Zielinska & Nowak, 2014). Controlling the lipid oxidation of PUFAs in facial cream is thus essential. As for lipid oxidation in foods, *F. vesiculosus* extracts could be a solution for this.

1.2.   Hypotheses, aim and objectives of the thesis

The overall aim of this PhD project is to develop new high value ingredients derived from the Nordic brown alga *F. vesiculosus* to be used as antioxidants by the food, cosmetic and pharmaceutical industry. In order to fulfil this task the work is divided into two main parts according to Fig. 1.1, i.e. "Extraction and characterization" (Part I) and "Application" (Part II).
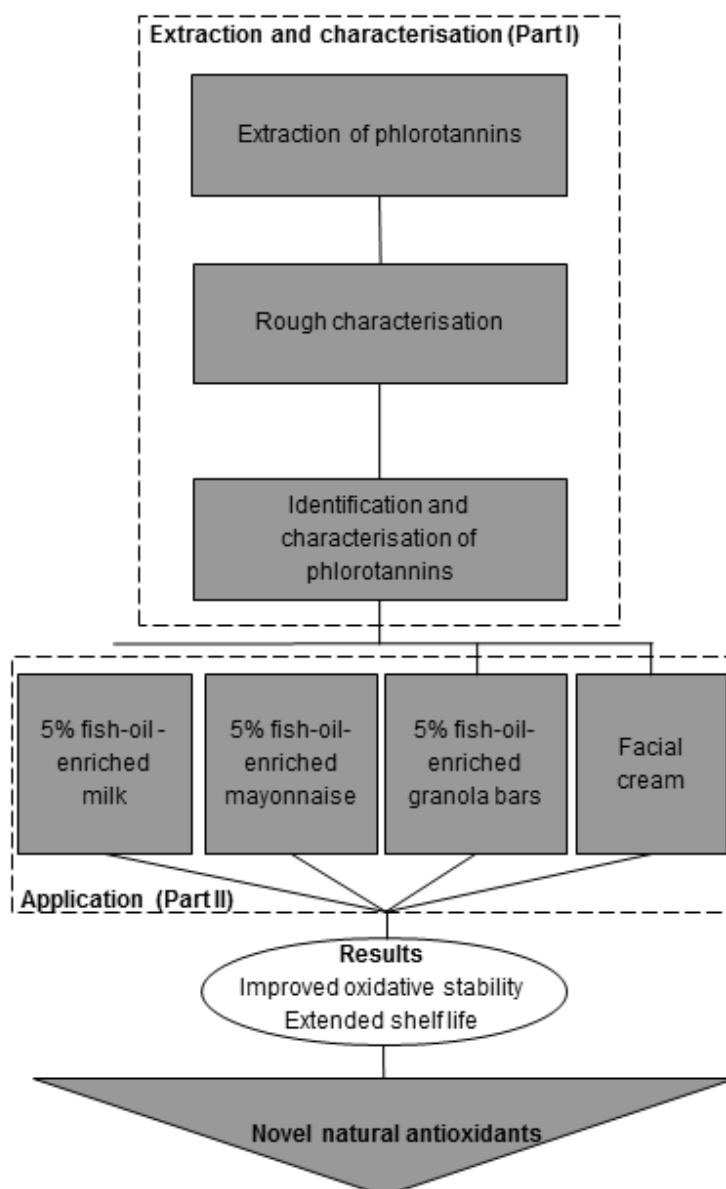
Fig. 1.1. Overview of the full PhD study including the two parts (Part I and Part II)

The PhD work tested the following four hypotheses (**H1-H4**):

- Different methods can be used to extract phlorotannins from *F. vesiculosus*. The type of extraction method used affects the composition and antioxidant activity of these extracts (**H1**)
- Even though, both antioxidants and prooxidants will be co-extracted, the major polyphenolic compounds, phlorotannins, are the main contributors to the antioxidant activity of *F. vesiculosus* extracts (**H2**)
- The phlorotannins contribute differently to the overall antioxidant activity of the extracts due to structural differences (**H3**)
- The *F. vesiculosus* extracts contain a wide range of antioxidants making them multifunctional. The extracts are therefore able to work efficiently in different food and skin care products (**H4**)

The hypotheses lead to the following three main objectives:

1. Extraction, identification and characterisation of phlorotannins from Nordic brown alga *F. vesiculosus*, including determination of the antioxidant contribution of specific phlorotannins
2. Characterisation of extracts by determining in vitro antioxidant activity and identifying the antioxidant and prooxidant substances in *F. vesiculosus* extracts
3. Testing the extract in different foods and one skin care product to find potential application for these

1.3.   Tasks and prerequisites

The project was partly funded by Nordic Innovation and has been associated with the strategic "Nordic Marine Innovation programme" project "Novel bioactive seaweed based ingredients and products", which was a collaboration between Icelandic, Norwegian and Danish partners from both research institutions and industry. For our part the work was in collaboration with the Icelandic company, Matís. Additional funding came from DTU.

According to the work package formulated by the syndicate of partners in the Nordic Innovation project, our task in collaboration with Matís was to carry out a characterization of Icelandic *F. vesicolosus* extracts and evaluate possible antioxidant contributors in the composition. Matís conducted solvent extracts and screened these successfully for antioxidant activity by *in vitro* assays before the extracts were delivered to us for our investigations, targeting applications for these extracts in different food and skin care product model systems. Hence, the extracts were tested in three types of foods and in one skin care product.

The PhD work additionally comprised a part taking the composition of the extracts into account when evaluating their antioxidant efficacy. Also, a method for identification and characterization of phlorotannins and their individual antioxidant activity was developed. Moreover, pressurized liquid extraction (PLE) was tested as a fast and environmental friendly alternative to solvent extraction of phlorotannins. PLE extracts were compared with solvent extracts by phlorotannin extraction efficacy and antioxidant properties.

The outcome will be a well-documented antioxidant effect of Nordic *F. vesiculosus* extracts both in *in vitro* studies and model systems and will disclosed examples of the application possibilities of seaweed extracts as natural antioxidants e.g. in the formulation of functional foods enriched with fish oil.

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Chapter 2: The Nordic brown algae

Seaweeds are known to contain unique compounds that can find many uses in consumer products. However, seaweed is still a highly underutilized resource in the Nordic Countries, even though cultivation of brown algae in Denmark has a potential of 5-15 tonnes harvested seaweed (dry weight) from 1 hectare (Alge Center Danmark, 2013). This great potential for utilization of seaweed has raised companies and researcher interest. In order to harvest and utilize brown algae we need to know more about how these grow and reproduce. Some brown algae are easily cultivated and produce high amounts of biomass, whereas other algal species grow slowly and are sensitive to environmental changes.

In this chapter the characteristics, reproduction and growth of brown algae will be outlined. The focus will be mainly on the Nordic alga *F. vesiculosus,* the target species of this PhD work. Furthermore, the possibilities within cultivation and harvesting, and also the utilization potential of *F. vesiculosus* will be discussed.

2.1. Brown algae

Brown algae have recently received interest from researchers due to their large variety of bioactive compounds and nutritional value (Hata et al., 2001; Kim et al., 2009; Holdt & Kraan, 2011). Brown algae are typically associated with the northern and cooler waters (Strömgren 1977). The most common brown algae in the North Sea are bladder wrack (*Fucus vesiculosus)*, toothed wrack (*Fucus serratus*) and sugar kelp (*Saccharina latissimi*).

Fucus vesiculosus has a leather-like surface with gas vesicles. This species dominates intertidal, typically sheltered, waters with rocky bottom (Moyse & Nelson-Smith, 1962). It is known for the air bladders, which are found in pairs on the blades as shown in Fig 2.2. These air bladders make *F. vesiculosus* float in the water and ensure e.g. efficient placing of the blades in the water to improve photosynthesis.

A *F. vesiculosus* plant comprises (Fig. 2.2), a holdfast (1), which is a root-like structure that ensures adhesion to the seabed, a stipe (2), which is the connection between the holdfast and the leaf-like blades, and the blades (3), with air bladders (4). At the tip of the blades swallen areas, the receptacles (5) with conceptacles (6) are placed.
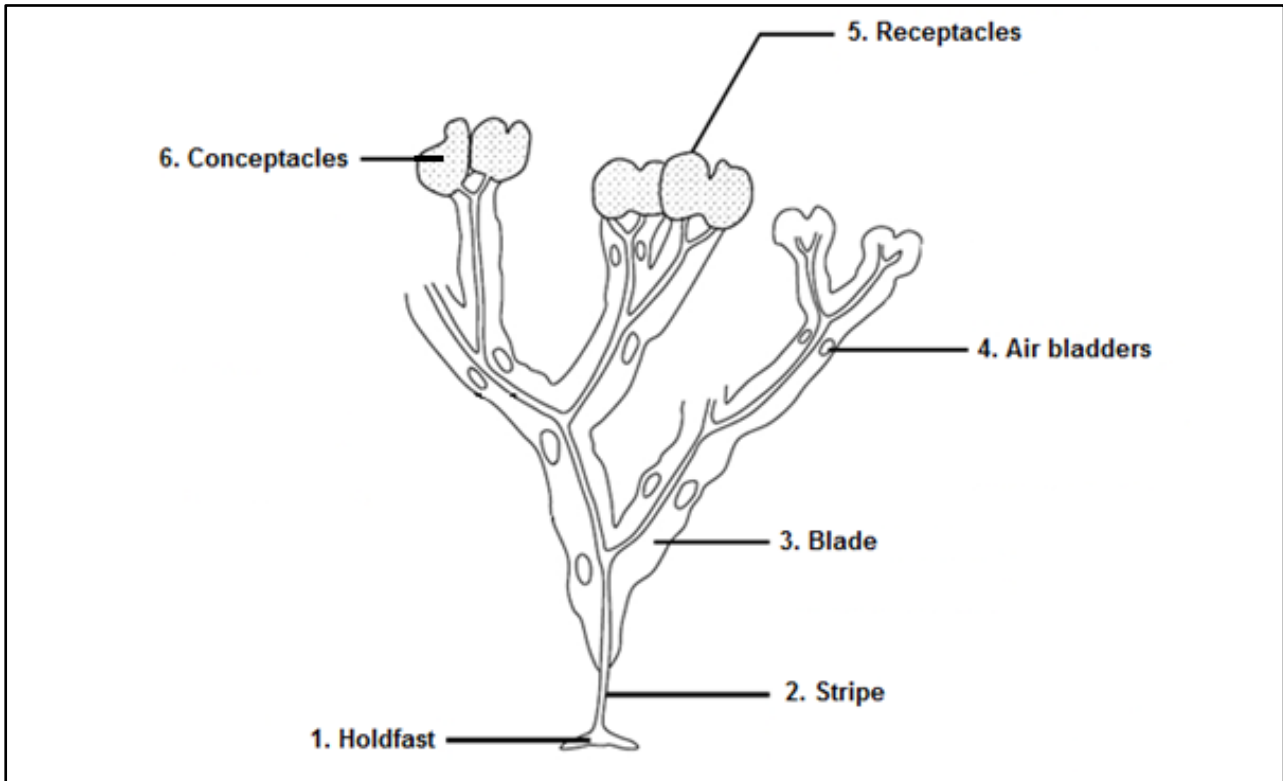
Fig. 2.2. Structure of a mature Fucus vesiculosus plant. 1) holdfast, 2) stripe, 3) blade, 4) air bladders, 5) receptacles and 6) conceptacles (Modified from Raven et al., 1992)

2.1.1. Reproduction and growth

Brown alga life cycle

The conceptacles, which appear as dots on the receptacles contain the reproductive material (Fig. 2.2-6). Most *Fucus* species are dioecious species with each sex being found on different plans. Others are monoecious with both sexes occurring on one plant in the same conceptacles. *Fucus vesiculosus* is diploid (2n) and meiosis takes place before gametes are formed resulting in only one set of chromosomes (1n). *Fucus vesiculosus* is monoecious but has the sexes in separate conceptacles. Eggs and sperm are released from the respective conceptacles and pheromone mediated fertilization takes place. The zygote (fertilized egg) begins to divide and eventually becomes a sporophyte which is the young diploid (2n). The North Sea *F. vesiculosus* reproduces once a year. The reproductive phase ends in June where the gametes are released into the water and becomes germinating sporophytes (Denny & Shibata, 1989; Bell, 1997; Pearson & Serrão, 2006). The release of gametes is controlled by water temperature and day length.

Growth conditions

Once the sporophytes are formed they attach to the seabed within a few meters from the parent plant and grow into sexual mature individuals (Coyer et al., 2006). Rocks, pebbles, boulders, mussel shells are preferable settling materials and stimulate germination. *Fucus vesiculosus* has apical meristem, so growth occurs only at the fertile areas of the tip of the plant. An adult *F. vesiculosus* plant needs to be attached to grow. However, detached *F. vesiculosus* specimens float due to their air bladders and are capable of reattaching to the seabed far away from the parental plant (van den Hoek, 1987). Young *F. vesiculosus* are fragile plants and when exposed to waves and storms they detach easily and float to start vegetation in another place (Ottesen, 2015). *Fucus vesiculosus* has been found to be smaller and less branched in waters with increasing physical disturbance, e.g. waves. Therefore, *F. vesiculosus* has optimal growth in sheltered or moderately exposed areas (Kalvas & Kautsky, 1993).

Abiotic factors, such as water temperature, available nutrients, light and salinity affect the growth of brown algae. The annual growth of the Nordic brown algae is generally increasing from January to June followed by a decrease from July to September. The Nordic alga *F. vesiculosus* has low growth in the winter period until January, where the water temperature is very low and there is a limited amount of daylight (Strömgren, 1986). Generally, *Fucus* species are not among the fastest growing brown algae. Strömgren (1986) found that the growth rate of Norwegian *F. vesiculosus* ranged from 1 to 12 µm/h, having the highest growth rate in June and the lowest in December. Strömgren (1986) suggested that this growth rate pattern was due to nitrogen accumulating in the *F. vesiculosus* thallus during winter and subsequent use of this nitrogen reserve during summer. In the late summer the ambient nitrogen reserve is used up mainly by fast growing microalgae, and the growth rate becomes nitrogen limited and hereafter the growth rate of *F. vesiculosus* reaches minimum, until the plant can start accumulating nitrogen from the surrounding water again.

Growth of *F. vesiculosus* is furthermore influenced by salinity and there is a general preference for high salinity. However, *F. vesiculosus* is able to adapt to and grow at different salinity levels ranging from the high salinity in the Norwegian Sea (34 psu) to the very low salinity (3.5-4 psu) in the Gulf of Bothnia and Gulf of Finland (Gylle et al., 2009). This is unique for *F. vesiculosus* since only few brown algal can survive such low salinities. However, the seaweed is also stressed by this condition, which is obvious from the dwarf-like morphology (Kalvas & Kautsky, 1998).

2.2. Cultivation and harvesting possibilities

There are two main types of cultivation, which at the moment are carried out on other species than *F. vesiculosus*, i.e. onshore cultivation, where cultivation is established in tanks or similar and offshore cultivation, where the cultivation is placed in the ocean. The harvesting procedure is very

important for regrowth and reproduction of *F. vesiculosus*, therefore this aspect should also be considered in a cultivation site of *F. vesiculosus*. In the following the two possible methods for cultivation of *F. vesiculosus* will be outlined. Furthermore, the harvesting approaches in cultivation of *F. vesiculosus* will be discussed.

2.2.1. Cultivation of brown seaweed in Nordic countries

To produce high quality seaweed based products from *F. vesiculosus* for biochemical utilization, cultivation and production should be considered. Production of brown seaweed is still a new discipline in Denmark as is also the case in other Nordic countries. Although several attempts have been made to introduce sophisticated technology for seaweed cultivation in tanks on land, none of these have attained commercial viability to date.

Onshore cultivation

There have been many attempts to cultivate seaweed onshore in tanks. A convenient technique is land-based tank cultivation of free-floating sporophytes of e.g. *Laminaria* agitated by air. These tanks allow high density of about 10 kg/m^2 (fresh weight) since the air circulation brings the seaweed to the water surface at intervals to allow photosynthesis. A high density and continuous circulation of the seaweed in tanks reduces fouling of unwanted epiphytes. A reduction of epiphytes can if needed be induced by short day treatment of the tanks by using blinds to simulate night and limit daylight to 8 h during the summer period. A high growth rate of the seaweed is still maintained under these conditions (review by Bartch et al., 2008).

Cultivation in tanks could be one option for cultivating *F. vesiculosus*. However, this technique could be costly due to the maintenance, energy consumption of pumps, temperature control, and addition of nutrients. Therefore, there is a huge interest in moving cultivation sites offshore to open water in order to lower the cost and "save space" on land and generally to secure a more sustainable production.

The major difficulties in the development of sustainable techniques offshore are, 1) the rough environmental conditions, which put the seaweed under stress that might result in reduced biomasses compared to tank cultivation, and 2) the less control of other parameters/variables, i.e. light, nutrients and temperature.

Offshore cultivation

Open water cultivation of seaweeds, attached to longlines is a rather low-tech method to cultivate seaweed. This approach is being used on a large scaling in e.g. Asia. Similar cultivation attempt have been done at Hjarnø Hage in Horsens fjord in Denmark (Fig. 2.3).
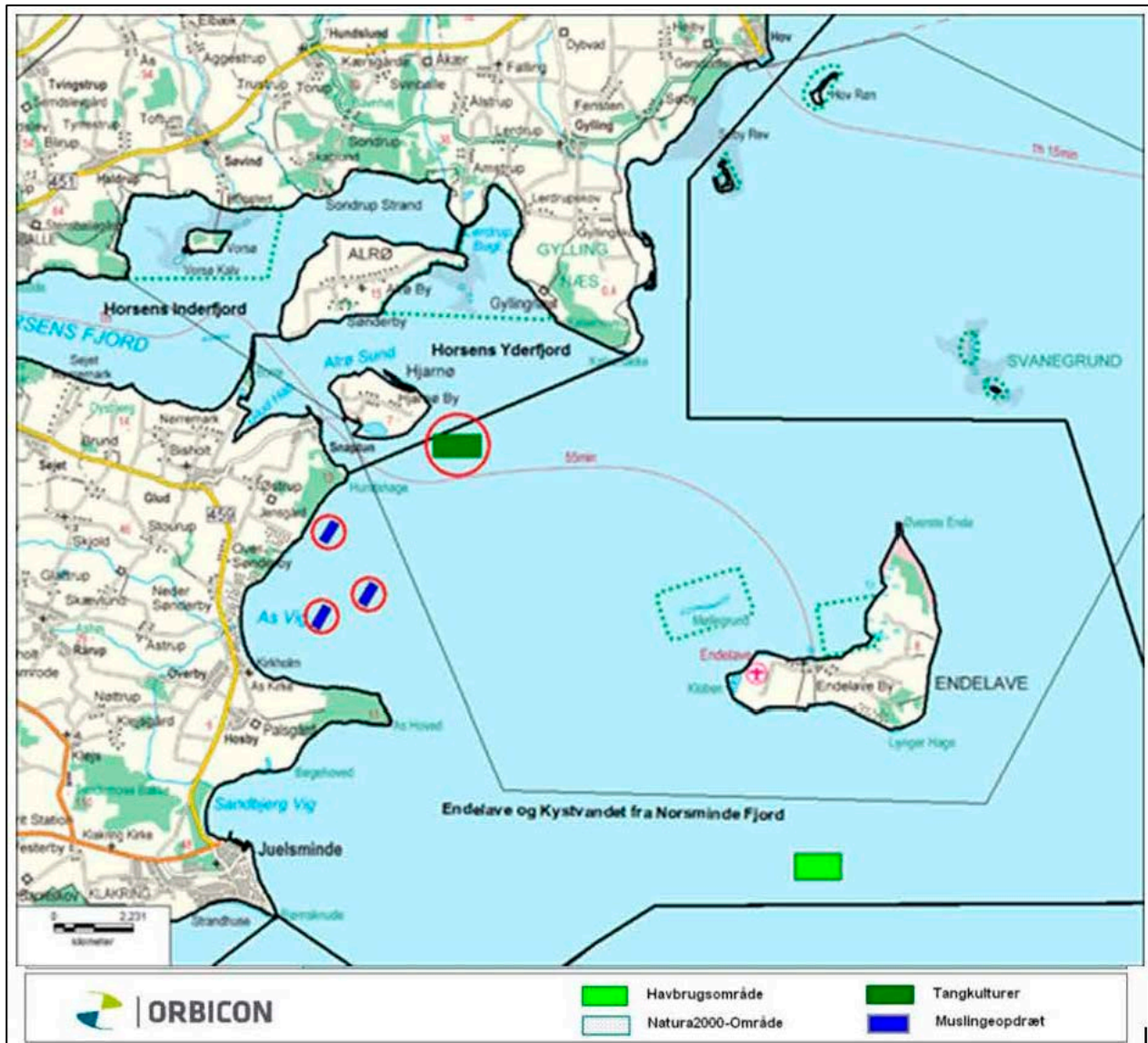
Fig. 2.3. Overview of Hjarnø combo farming at Horsens outerfjord. The light green is the fish farm, blue is the muscle farm and the dark green is the seaweed, S. latissimi

This is the first certified organic combo farming (fish/mussels/seaweed) in Denmark, established by Hjarnø Havbrug to improve the water environment while producing diverse products (Hjarnø, 2015; NaturErhvervstyrelsen, 2015). The seaweed plantation at Hjarnø Hage relates to *Saccharina latissima* also known as sugar kelp. The seeds (spores or gametophytes) from this seaweed are settled on strings at a land-based hatchery and placed in the waters outside Hjarnø Hage where they grow in the natural environment of the surrounding water. The harvesting outcome for such a production is 1-16 kg fresh weight m^{-1} cultivation line (Peteiro & Freire, 2013; Marinho et al., 2015).

While *S. Latissima* is relatively easy to grow in open water, *F. vesiculosus* is more sensitive. *Fucus vesiculosus* prefers intertidal, sheltered places where it can adhere to stones or similar in the

seabed. These conditions are difficult to accommodate in an open water production, and for this species specifically the settling of germinating sporophytes on e.g. ropes have not been fully developed for up-scaling yet.

At the Danish Island Endelave outside Horsens fjord (Fig. 2.3), Endelave Seaweed Aps (Endelave Seaweed, 2015) has been aiming for creating such an environment to promote the growth of *F. vesiculosus*. The company has experimented with rocks, rope and net for establishing more kelp forest and increasing the production of *F. vesiculosus* at Endelave. Their findings indicate that the net gives good attachment of both sporophytes and vegetative plants (Ottesen, 2015). In the Danish straits where both Horsens outer fjord and Endelave are located the salt content is approximately 20 psu, which is suited for *F. vesiculosus* (Sørensen, 2012).

Sum up

The growth conditions required to get the optimal yield of *F. vesiculosus* is not suited for open water offshore cultivation. On the other hand an onshore cultivation is costly. It is possible though to create the right conditions on the seabed at intertidal, sheltered place with relatively high salinity for wild population of *F. vesiculosus* to settle and grow to be harvested and utilized for production of high quality seaweed based products.

2.2.2. Harvesting

There are two typical harvesting forms; trawling or manually. One is done from boats or larger vessels and is normally only used for wild harvesting in open water. The other is done with scissors/knifes or similar by hand, typically at onshore cultivation sites or wild harvesting at low water near the coast.

In order to optimize the outcome of *F. vesiculosus* and maintain a sustainable production, the harvesting should be gentle in order for the seaweed to survive and continue its growth. However, since *F. vesiculosus* only grows from the tip, the growth zone is cut off during harvesting. Harvesting of *F. vesiculosus* is typically carried out by lifting the plants up from the water (still attached to the stone or similar) and cut near the apical tip (Ottesen, 2015). This harvesting method does not kill off the seaweed but stresses it to produce more fronds merging from the same holdfast. This holdfast proliferation can be considered vegetative reproduction (Ruuskanen & Bäck, 1999). These new plants can fall off when the water gets rough from a storm and float with the water to possibly attach to the seabed at another site and start to grow (vegetative reproduction). Endelave seaweed Aps harvests *F. vesiculosus* twice a year: in May/June and November/December (Ottesen, 2015).

2.3. Utilization possibilities of *F. vesiculosus*

Fucus vesiculosus is a potential source of biochemical compounds exhibiting a broad spectrum of biological activities. This PhD thesis focuses on exploring the potential of utilizing *F. vesiculosus* for production of novel natural antioxidants to be used by the food, cosmetic and pharmaceutical industry. Since *F. vesiculosus* is rich in antioxidative polyphenolic compounds called phlorotannins (Koivikko et al., 2005; Wang et al., 2009), the extraction of these compounds is of primary interest.

In food systems, extracts from *F. vesiculosus* has the potential to inhibit lipid oxidation and enhance the oxidative stability (Wang et al, 2009; Halldorsdottir et al., 2014). This functionality can be extrapolated to both skin care and medical products to protect active lipids. Moreover, the functionality of phlorotannins, i.e. anti-aging and anti-inflammatory activity, makes these compounds well suited in skin care products (Dutot et al., 2012). The antioxidant activity and application possibilities of *F. vesiculosus* extracts will be discussed in Chapter 4.

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Chapter 3: Lipid oxidation

Lipid oxidation leads to development of undesirable flavours and odours and is the main reason for quality changes and rancidity in products rich in unsaturated fatty acids. Lipid oxidation can be divided into three categories depending on how it is initiated; autoxidation, photooxidation and enzymatic oxidation. This chapter will mainly focus on the aspects of autoxidation in oil-in-water (o/w) emulsions, since the PhD work considers foods or preemulsions added to foods and skin care emulsions. The chapter includes an overview of the formation of primary and secondary volatile oxidation products during oxidation of especially polyunsaturated fatty acids (PUFA), since the foods contain fish oil (FO) rich in eicosapentaenoic acid (C20:5 (n-3), EPA) and docosahexaenoic acid (C22:6 (n-3), DHA) and the skin care product is rich in linoleic and linolenic acid. There will be a special focus on factors influencing these processes in foods and skin care products similar to those used in the thesis.

3.1. Autoxidation; initiation, propagation and termination

The prerequisites in order for autoxidation to start are the presence of unsaturated lipids (LH), initiators (In•) and oxygen (Fig. 3.1).
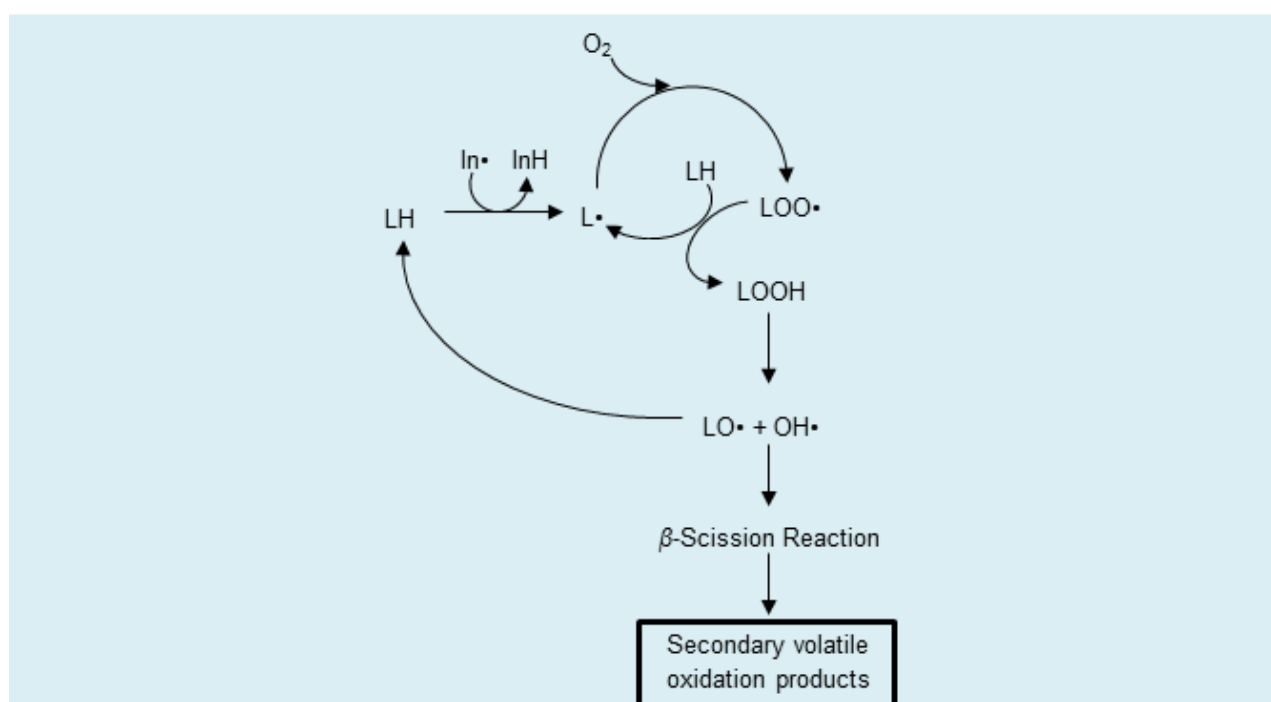
Fig. 3.1. Schematic diagram of autoxidation of unsaturated lipids. LH: Unsaturated lipid, In•: Radical initiator, L•: Lipid alkyl radical, LO•: Lipid alkoxyl radical, LOO•: Lipid peroxyl radical, LOOH: Lipid hydroperoxide
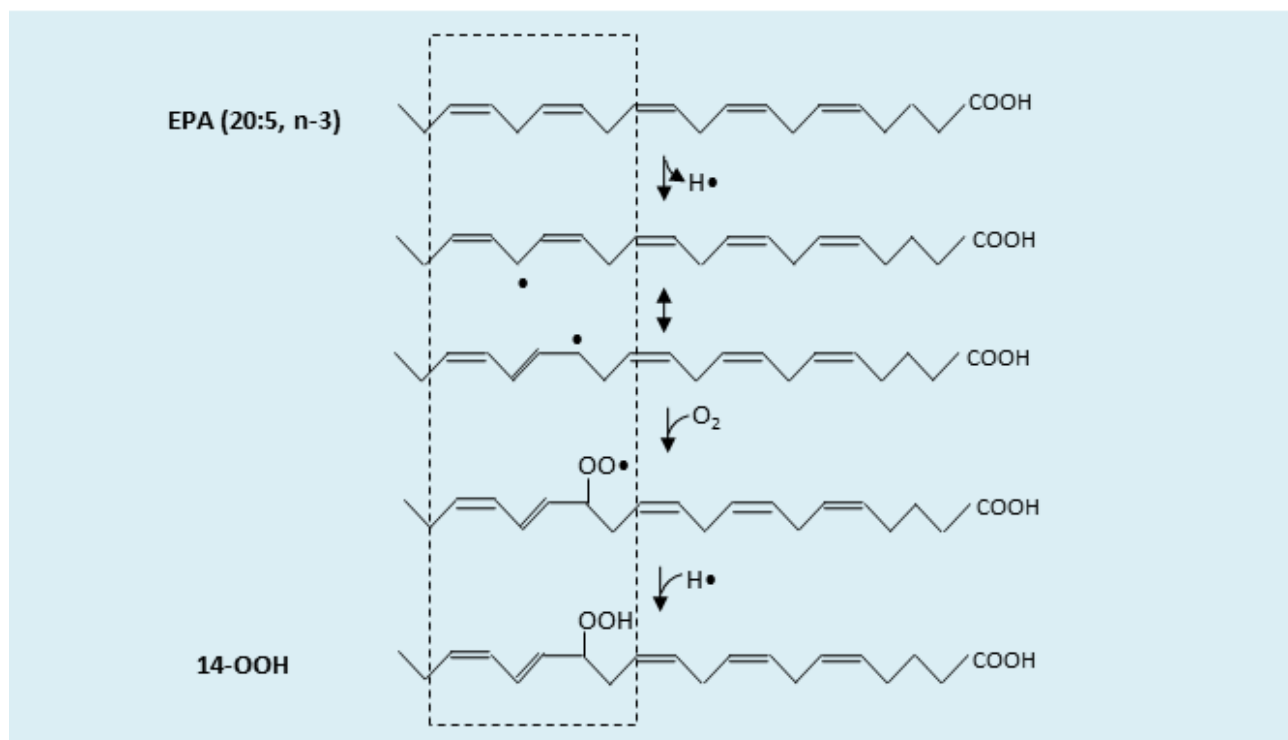
Fig. 3.2. Example of resonance stabilization and formation of conjugated double bond in formation of hydroperoxides from EPA (20:5(n-3))

The propagation of autoxidation is terminated when two free radicals form a non-radical product such as a fatty acid dimer, trimers and oligomers, and terminate the process. Termination is not as important in foods and skin care products rich in unsaturated fatty acids since these products are already rancid before significant termination reaction has taken place (Frankel, 2005).

3.2. Volatile secondary oxidation products

The development of undesirable flavours and odours in food and skin care products originates from the decomposition of lipid hydroperoxides (LOOH) (Fig. 3.2). Whereas lipid hydroperoxides are tasteless and odourless oxidation products, secondary volatile oxidation products are responsible for the unwanted quality changes in these types of products. Many volatile products are detectable by humans at parts per million and even parts per billion thresholds (Labuza & Dugan, 1971).

Lipid hydroperoxides are decomposed into lipid alkoxyl radicals (LO•) through hemolytic cleavage and further to low molecular weight, volatile compounds by β-scission reaction as illustrated in Fig. 3.3. These low molecular weight volatile secondary oxidation products are responsible for the rancidity of products rich in unsaturated fatty acids (Frankel, 2005).
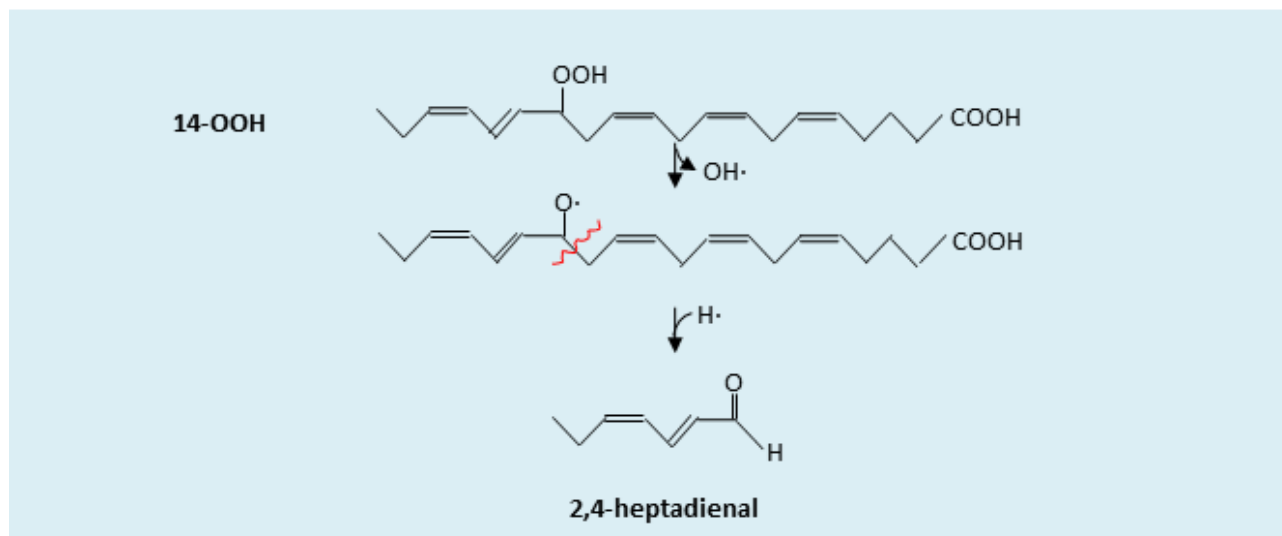
Fig. 3.3. β-scission reaction (marked as a red scission) of 14-OOH (hydroperoxide) involved in the formation of short chain volatile secondary oxidation product, 2,4-heptadienal, from oxidation of EPA (20:5(n-3))

In table 3.1. some volatile secondary oxidation products associated with oxidation of PUFA are listed. All these compounds have been found in products or ingredients similar to those used in this PhD work (FO-enriched milk: Venkateshwarlu et al., 2004a; FO-enriched mayonnaise: Hartvigsen et al., 2000; FO-enriched energy bars: Horn et al., 2009; Almonds: Salcedo and Nazareno, 2015). Complex mixtures of volatile secondary oxidation products such as aldehydes, ketones, alcohol, alkanes and alkenes are formed during lipid oxidation depending on the structure of the original lipid hydroperoxide. For example, propanal, 2-hexenal and 2,4-heptadienal are specific products formed from PUFAs from the n-3 family. In general, aldehydes and ketones have low threshold values and thus can have high impact on the flavor changes in oxidized products (Frankel, 2005).

When monitoring lipid oxidation over time, a lag phase with slow accumulation of lipid oxidation products is typically observed. This lag phase is the result of slow formation of free radicals prior to lipid hydroperoxide accumulation and *β*-scission reactions into low molecular weight volatile secondary oxidation products. The lag phase can be extended by the presence of radical scavenging antioxidants until these antioxidants are used. When decomposition of lipid hydroperoxides begins and/or antioxidants are used up, lipid alkoxyl radicals (LO•) are formed and attack additional unsaturated fatty acids, leading to an exponential increase in oxidation rate. This exponential increase in oxidation products can occur at very different rates, indicating that the overall lipid oxidation does not follow first order kinetics.

Table 3.1. Volatile secondary oxidation products related to lipid oxidation products or ingredients similar to the once used in the PhD work (FO-enriched milk, mayonnaise and energy bars as well

as almonds (almond oil used in facial cream). The papers (I to V) in which the volatiles have been found are stated.

Group of oxidation products	Oxidation products	Found in*	Reported in Paper I to V
Aldehydes	Pentanal	Milk, mayonnaise, energy bars, almonds	I, II, III, IV, V
	Hexanal	Milk, mayonnaise, energy bars, almonds	I, II, III, IV, V
	Heptanal	Milk, mayonnaise	I, V
	Octanal	Milk, mayonnaise	I, III, IV
	Nonanal	Mayonnaise, energy bars, almonds	I, II, III
	Decanal	Milk, mayonnaise	III
	2-Pentenal	Milk, mayonnaise, energy bars	II, III
	t-2-Hexenal	Milk, mayonnaise	I, II, III, IV
	2-Heptenal	Milk, mayonnaise, almonds	III
	c-4-Heptenal	Milk, mayonnaise	IV
	2,4-Heptadienal	Milk, mayonnaise, energy bars	I, II, III, IV
	t,c-2,6-Nonadienal	Milk, mayonnaise, energy bars	IV
	Benzaldehyde	Milk, mayonnaise	III, IV
Alcohols	3-Methyl-1-butanol	Mayonnaise	I
	1-Pentenol	Milk, energy bars	I, III, IV
	1-Penten-3-ol	Milk, mayonnaise, energy bars	I, II, III, IV
	1-Octen-3-ol	Milk, mayonnaise, energy bars	III, IV
Ketone	1-Penten-3-one	Milk, energy bars	I, II, III, IV
Furan	2-Ethylfuran	Milk, mayonnaise	II, III, IV
	2-Pentylfuran	Mayonnaise	III, IV

*According to: Venkateshwarlu et al. (2004a), Hartvigsen et al. (2000), Horn et al. (2009) and Salcedo & Nazareno (2015)

3.3. Lipid oxidation in oil-in-water emulsions

In this thesis o/w emulsions were studied. Milk, mayonnaise and facial cream are all o/w emulsions, and granola bars were added a preemulsion of 70% fish oil-in-water (FO/w). These systems, except skin care products, have previously been used as models for monitoring lipid oxidation as shown in Table 3.1. Therefore the focus in the following section will mainly be on oxidation in o/w emulsions or these added to foods as a preemulsion.

Food and skin care products usually exist as emulsions where different factors play a role in the lipid oxidation process. An emulsion is one phase dispersed throughout another phase in which it is immiscible, such as oil and water. In the emulsion the dispersed phase exists as droplets in the other continuous phase, stabilized by surface-active molecules adsorbed at the interface. Emulsions can be conveniently divided into three different regions: the continuous phase, the

interfacial region, and the interior of the droplets (McClements & Decker, 2000). Emulsions are thermodynamically unstable and require stabilizers, such as emulsifiers, to maintain their physical structure and prevent gravitational separation, flocculation and coalescence.

A system comprising oil droplets dispersed in a water phase is an o/w emulsion (e.g. milk or mayonnaise). Conversely, a system comprising water droplets dispersed in an oil phase is a water-in-oil (w/o) emulsion (e.g. butter). The initial step of lipid oxidation in these emulsions takes place at the interface between the oil and water phases (McClements & Decker, 2000). During the emulsification process in production of e.g. mayonnaise, the interfacial area between oil and water is substantially increased. This interfacial area increases the potential contact area between oil and possible initiators in the continuous, aqueous phase. Therefore, o/w emulsions are usually more prone to lipid oxidation compared to bulk oil, due to a high surface area, which increases interactions between the lipids and prooxidative substances in the water phase. However, lipid oxidation in o/w emulsion systems has the opportunity to be controlled in both the oil, aqueous and interfacial phase. Strategies to hinder lipid oxidation in o/w emulsions used in this PhD thesis will be discussed in Chapter 4.

3.4. The food and skin care product model systems used in the thesis

In this thesis both food model systems and a skin care model system were used to study the efficacy of seaweed extracts towards inhibition or reduction of the lipid oxidation process. These systems are presented below. They are all emulsions (or products added emulsions) with high complexity and contain, in contrast to bulk oil, an array of ingredients. More details on the factors influencing lipid oxidation in these systems will be discussed in section 3.5.

3.4.1. Fish-oil-enriched food products

Lipid oxidation in FO-enriched products is more pronounced due to the highly unsaturated fatty acid content in FO, such as EPA and DHA, two major omega-3 long chain polyunsaturated fatty acids (n-3 LC PUFAs) in FO. These n-3 LC PUFAs have shown to e.g. reduce the incidence of important human disorders including atherosclerotic cardiovascular disease, cardiac sudden death, stroke, and asthma and furthermore play an important role in brain functioning and development in infants (Broughton et al., 1997; Horrocks & Yeo, 1999; Barnham et al., 2004; Calder, 2006; Saito et al., 2008; Covert, 2009). The reason for enriching foods with FO is to increase the intake of these healthy n-3 LC PUFAs.

Enrichment with FO, rich in these n-3 LC PUFAs, leads to an increase lipid oxidation of the product and fast development of undesirable off-flavours (Jacobsen et al., 2000; Let et al., 2003; Let et al.,

2007a). Controlling the oxidation in these types of product are widely studied and the development of a strategy to hinder fast oxidation has come closer (Jacobsen, 2015).

Milk

Milk is an o/w emulsion, which mainly consists of water (whole milk, approximately 87.2% water). The fat content of whole milk is 3.5% (95% triglycerides) while protein is 3.5% (80% casein and 80% whey protein) and lactose is 4.9%. Milk also contains minerals such as calcium, iron and cupper.

The oxidative stability and also strategies to limit lipid oxidation in FO-enriched milk have been studied by e.g. Let et al. (2003; 2007a; 2007b), Sørensen et al. (2007), Venkateshwarlu et al. (2004a; 2004b) and Alemán et al. (2015). Let et al. (2007a) found higher stability of FO-enriched milk when FO was in emulsion. Furthermore, Let et al. (2007b) tested processing conditions (temperature and pressure) in production of FO-enriched milk. They found that the best oxidative stability was obtained when FO was homogenized at high temperature (72°C) and at high pressure (22.5 MPa). Sørensen et al. (2007) found that at these processing conditions the incorporation of the FO and its protection by milk proteins at the oil-water interface was optimal.

Mayonnaise

Mayonnaise is just like milk a complex food where several factors may affect the oxidation. Mayonnaise usually consists of oil, water, and vinegar or lemon juice, egg yolk, stabilizing agents and flavouring compounds. Traditionally, mayonnaise is made from emulsification with egg yolk to incorporate 60-80% oil in an o/w emulsion.

FO-enriched mayonnaise has been studied mainly regarding the influence of ingredients and conditions such as iron, pH and emulsion structure on the oxidative stability and development of off-flavours and odours (Hartvigsen et al., 2000; Jacobsen et al., 1999; Jacobsen et al., 2000; Jacobsen et al., 2001a; Jacobsen et al., 2001b; Alemán et al., 2015). Studies of FO-enriched mayonnaise showed that metal catalysed lipid oxidation is an important factor influencing the oxidative stability of this product due to the low pH and the presence of egg yolk which is rich in iron (Jacobsen et al., 1999; Jacobsen et al., 2001a; Jacobsen et al., 2001b).

Granola bars

Although rancidity has been studied in many types of foods, little research has been done on low-moisture baked foods, and food products with a water activity (a_w) below 0.5 ($a_w > 0.5$) whose shelf-life is primarily limited by lipid oxidation and non-enzymatic browning more than microbial growth like biscuits, crackers or granola bars (Labuza et al., 1970).

Granola bars are mainly made from wheat flour, rolled oats, sugar, dried fruits and fat. The ingredients are mixed together and baked. Typically, the bars are atmospherically packed in sealed foil without light. This grain-based product is a popular ready-to-eat snack. The fat in granola bars mainly consists of saturated fat, which makes this type of product a good candidate for replacing some of the saturated fatty acids with unsaturated fatty acids and thereby improving the nutritional profile.

A few studies have previously dealt with lipid oxidation in FO-enriched granola bars (Nielsen et al., 2009; Horn et al., 2009; Hughes et al., 2012). Hughes et al. (2012) considered the consumer acceptance when some of the saturated fat in granola bars was replaced with FO. They found that the sensory panel could not detect fishy flavour in the control samples without any FO and samples with 20% FO replacement (corresponding to 1% FO in the bars). Nielsen et al. (2009) studied the oxidative stability of 5% FO-enriched granola bars when FO was added as either neat FO, as a FO/w emulsion with 10% sodium caseinate (Na-cas) as the emulsifier or as microencapsulated FO. The results showed that the oxidative stability of the bars increased in the order: neat oil < FO/w emulsion < microencapsulated FO.

3.4.2. Skin care products

Facial cream

Facial cream is a well-known skin care product, which is used daily in the normal household. This type of product contains a wide range of active compounds, which improve and strengthen the skin. Especially the content of natural oils rich in unsaturated n-6 and n-3 fatty acids such as linoleic and α-linolenic acid (ALA), have important functional skin improving properties both against dry and oily skin (Sathe et al., 2008; Zielinska & Nowak, 2014). These unsaturated fatty acids are as mentioned susceptible to lipid oxidation and oxidation can lead to undesirable off-odours in the product. To ensure the maximum durability of skin care products, they should be stored in a cool, dry place, away from direct sunlight and extreme temperatures. However, this is not always the case. Products like this are sold all over the world and the storage conditions are not as controlled as for food. Therefore products like facial cream should be able to withstand extreme conditions like light and high temperatures and addition of antioxidants could be a solution to maintain high quality products even under extreme conditions.

3.5.   Factors influencing lipid oxidation in foods and skin care emulsions

Lipid oxidation is a tricky player in the quality control of foods and skin care products. The strategies currently carried out by the industry to counteract lipid oxidation include the use of different packaging techniques, i.e. modified atmosphere packaging, low-temperature storage and

sunlight protection, or addition of antioxidants. Despite these efforts, the products containing PUFAs are still subject to risks of unpredicted development of lipid oxidation during storage. In the following section, factors influencing lipid oxidation in food and skin care emulsions will be outlined and discussed.

3.5.1. Ingredients

Fatty acid composition

The products studied in this PhD project all contain some degree of unsaturated fat. The unsaturation of the lipids present in the food or skin care emulsion highly influences the degree of lipid oxidation, since oxidation increases with increased unsaturation (Frankel, 2005). Addition of FO rich in PUFAs to the food products will therefore further enhance lipid oxidation and decrease the shelf life of these types of products.

Water

The water content in the different products is highly variable (5-87.2%). This water can take two forms; 1) free or available water, or 2) bound water. Bound water is typically bound to proteins or carbohydrates. The available water or a_w of a system influences the rate of lipid oxidation. In low-moisture foods the limited mobility of components like antioxidants results in high lipid oxidation rates compared to the rate at intermediate water activities (Labuza et al., 1972). For many of the chemical reactions such as lipid oxidation, a decrease of $a_w < 1.0$ can initially cause an increase in reaction rates as substrates such as transition metals becomes more concentrated. However, a further decrease in a_w will significantly lower the reaction due to diffusion limitations.

Emulsifiers

Emulsifiers are either added or naturally present in these products. Their role is to form a stabilizing film around the oil droplets to make a stable emulsion and preventing the oil droplets from aggregating or from gravitational separation.

Proteins have been extensively used for stabilizing emulsions due to their emulsifying properties (McClements, 2004). Na-cas is an ingredient widely used due to its functional properties, which include emulsification, water and fat-binding, thickening and gelation (Kinsella & Morr, 1984). This ingredient is a mixture of four caseins (α_{s1}-, α_{s2}-, β-, and κ-casein), which, in aqueous solutions, form complexes and aggregates with a wide range of molecular weights (Singh et al., 2003). Nevertheless, the protein-stabilized emulsions are highly sensitive to environmental conditions, such as the pH value, ionic strength and temperature (Gu et al., 2004; Pallandre et al., 2007). For example, milk proteins provide good stability for emulsions at neutral pH due to a combination of

electrostatic and steric stabilization mechanisms (Dickinson et al., 1998). Charged emulsifying proteins can influence the lipid oxidation and will be discussed in section 3.5.3.

Ingredients such as egg yolk in the mayonnaise, which is traditionally used, as the emulsifying agent in this type of product, has been suggested to influence oxidation, due to its high content of iron. In egg yolk, the major proportion of iron is bound to the protein phosvitin (Causeret et al., 1991). However, when the egg yolk is incorporated into mayonnaise, the low pH (pH 4) is responsible for breaking the ion bridges between iron and phosvitin, which releases iron, and in turn enables it to participate in lipid oxidation (Jacobsen et al., 1999).

3.5.2. Prooxidants

The most prone prooxidant in food and skin care products is iron. Iron stems from ingredients such as egg yolk, raisins, wheat flour and water (Yeung et al., 2003). Water can be both antioxidative and prooxidative, since water can associate with lipids and protect them from oxygen and thereby preventing lipid oxidation. On the other hand, water can play a major role in lipid oxidation chemistry if reactions are primarily promoted by water-soluble prooxidant such as iron (Barden & Decker, 2015a).

Heat and light can also promote the formation of lipid hydroperoxides. If these two factors are controlled, the presence of transition metal ions from the ingredients has shown to be the most pronounced prooxidant. Ferrous ions (Fe^{2+}) have higher solubility and reactivity than ferric ions (Fe^{3+}) and are therefore more prooxidative (Halliwell & Gutteridge, 1990). Ferrous ions are converted to ferric ions during interactions with lipid hydroperoxides.

These reactive metals decompose lipid hydroperoxides into free radicals, where Mn^{n+} and $Mn^{(n+1)+}$ are the transition metals in their reduced and oxidized state as shown in scheme 3.1.

$Mn^{n+} + LOOH \longrightarrow Mn^{(n+1)+} + LO\cdot + OH^-$	*Reaction I*
$Mn^{(n+1)+} + LOOH \longrightarrow Mn^{n+} + LOO\cdot + H^+$	*Reaction I*
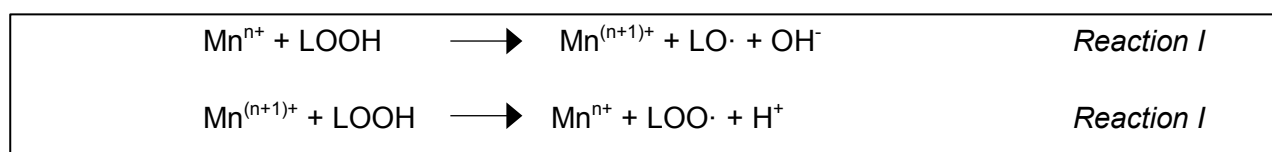
Scheme 3.1. Overview of metal initiation in lipid oxidation. M: Metal ion, LOOH: Lipid hydroperoxide, LOO•: Lipid peroxyl radical, LO•:Lipid alkoxyl radical (adapted from Frankel, 2005).

3.5.3. Physical structure and stability

The physical stability of an o/w emulsion is highly dependent on emulsifiers and droplet distribution. Oil droplets are distributed evenly throughout the product during emulsification and

emulsifiers are partitioning on the oil surface to create a physical barrier around oil droplets to maintain its physical structure.

Surface area and oil droplet distribution

A broad range of oil droplets from less than 0.1 to 20 µm in diameter can usually be encountered in food emulsions. The droplet size distribution of an emulsion is typically determined by light scattering techniques to create a histogram of droplet diameters (*D[3,2]*) (McClements, 2005). The droplet size influences the viscosity of a product, where small oil droplets give a more viscous product than larger droplets (Jacobsen et al., 2000). A high viscosity could influence e.g. the diffusion of prooxidant transition metals and thereby reduce their interactions with the oil phase (Sims et al., 1979). On the other hand, as lipid oxidation occurs at the interface between oil and water in o/w emulsions, small droplets increase the interfacial area resulting in interaction between metal ions and unsaturated lipids and/or lipid hydroperoxides which in turns leads to increased lipid oxidation.

The oxidative stability of FO enriched mayonnaise is highly dependent on the stability of the emulsion. Jacobsen et al. (2000) suggested that the structure of mayonnaise should be aimed at medium to large (*D[3,2]*) oil droplets (less viscous product) more than small droplets since the viscosity influences oxidation rate less than the size of the interfacial area, due to high amounts of iron in mayonnaise.

In contrast, Gohtina et al. (1999) found no correlation between oil droplet size and lipid oxidation, indicating the influence of other factors than oil droplet size on lipid oxidation. Let et al. (2007b) and Sørensen et al. (2007) found that when obtaining small droplets in milk by higher pressure homogenization, the distribution of protein at the interface and in the water phase, overruled the influence of surface area on lipid oxidation. Therefore, in milk, the distribution of protein influences the oxidative stability more than the surface area.

The relationship between physical structure and oxidative stability of low-moisture foods (crackers) has been studied by e.g. Barden et al. (2015b), using confocal imaging to determine the distribution of added oil. It was found that the added oil was evenly distributed throughout the crackers, and also that the oil was not highly associated with air and water. It was additionally confirmed that the protein (origin from the ingredients) formed protein bands, which did not surround the oil. Hence, there was no protective effect of protein on the added oil to hinder lipid oxidation. Overall, the interactions between the oil, water and protein in the low-moisture crackers added oil were very different from oil-in-water emulsions.

Surface charge

The electrical charge of emulsion droplets has strong influence on the interactions between prooxidant metals in the water phase and the lipids in the oil phase. Interfacial charge will either attract or repel prooxidant metals (+/-). A negatively charged surface might thus attract positively charged transition metals such as iron located in the water phase. This means that iron will be more associated with the lipid surface and therefore also the unsaturated fatty acids or lipid hydroperoxides and enhance lipid oxidation (McClements & Decker, 2000). This surface charge dependent oxidation has been confirmed in several studies (Mei et al., 1998a; Mei et al., 1998b; Tong et al., 2000).

The surface charge of the oil droplets and emulsion stability are highly dependent on pH, since emulsifying proteins become unstable when the pH is adjusted close to their pI (McClements, 2005). E.g. milk is a stable emulsion with negatively charged surface area of the oil droplets at pH of 6.5, which is higher than pI for the proteins (Fox & McSweeney, 1998; Walstra & Jenness, 1984). The fact that the surface charge of the oil droplets in milk are negatively charged may increase lipid oxidation since iron will be attracted to the surface and increase the interaction between prooxidative iron and unsaturated lipids and/or lipid hydroperoxides.

The surface charge can be modified by emulsifiers or by electrostatic layer-by-layer electrostatic deposition technique (Bouyer et al., 2011; Gu et al., 2004; Guzey & McClements, 2006; Liu et al., 2012; Pallandre et al., 2007), in which charged biopolymers are adsorbed onto oppositely charged droplets to form a stable membrane at the oil-water interface of the emulsion. Many studies have demonstrated that the stability of emulsions to environmental conditions can be improved by the formation of layer-by-layer emulsification (protein-polysaccharide complexes through covalent bonding) (Kato, et al., 1992; Oliver, et al., 2006; O'Regan & Mulvihill, 2010) or electrostatic interactions (Bouyer et al., 2011; Gu et al., 2004; Liu et al., 2012; Pallandre et al., 2007).

3.6.   References

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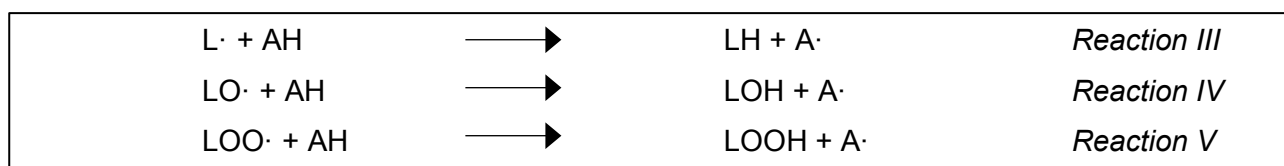
Chapter 4: Antioxidant strategies in food and skin care products

In food or skin care o/w emulsions, like the ones used in this thesis, lipid oxidation is one of the major contributors to the quality deterioration and loss of functional fatty acids. Antioxidant strategies can be used to increase the oxidative stability of these products prone to lipid oxidation.

4.1. Antioxidants and their mechanisms

Overall, antioxidants can be classified as primary or secondary antioxidants depending on their mechanisms. Moreover, some antioxidants can be referred to as multi-functional as they exhibit both primary and secondary antioxidant properties.

Primary antioxidants, also referred to as chain-breaking antioxidants, are able to react directly with free radicals by converting them to more stable, non-radical products. Hence, primary antioxidants play an important role in the initiation and propagation step of lipid oxidation since they can react with lipid alkyl (L·), alkoxyl (LO·) and peroxyl (LOO·) radicals (Scheme 4.1, reaction, III, IV and V) (Decker, 2002). Thereby, further decomposition into volatile oxidation products is hindered. Phenolic compounds with more than one hydroxyl group (-OH) are efficient primary antioxidants due to their ability to donate H-atoms to free radicals, creating relatively unreactive phenoxyl radicals. Synthetic phenolic compounds, i.e. butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), are efficient chain-breaking antioxidants and widely used as food preservatives. Furthermore, naturally occurring phenolic compounds such as tocopherol, ascorbic acid or caffeic acid are also used as chain-breaking antioxidants but are typically less efficient compared to the synthetic ones.

L· + AH	$\longrightarrow$	LH + A·	*Reaction III*
LO· + AH	$\longrightarrow$	LOH + A·	*Reaction IV*
LOO· + AH	$\longrightarrow$	LOOH + A·	*Reaction V*

Scheme 4.1. Reactions between primary antioxidants (AH) and lipid (L·), alkoxyl (LO·) and peroxyl (LOO·) radicals to form unsaturated lipids (LH), Lipid alcohol (LOH), lipid hydroperoxides (LOOH), and antioxidant radicals (A·)(adapted from Chaiyasit et al., 2007).

Secondary, or preventive, antioxidants work indirectly on limiting lipid oxidation. Several mechanisms including chelation of transition metals, singlet oxygen quenching (in photooxidation) and oxygen scavenging can be exhibited by these secondary antioxidants (Decker, 2002). Furthermore, some secondary antioxidants can regenerate primary antioxidants in a synergistic

manner, and thereby restore the antioxidant activity of primary antioxidants to ensure their continuous antioxidant activity. The ability of a secondary antioxidant to chelate metals is an important property for antioxidants in food systems, since metal-induced lipid oxidation is pronounced in these products. Synthetic ethylenediaminetetraacetic acid (EDTA) and caseinate are some examples of metal chelating antioxidants (Haahr & Jacobsen, 2008).

Addition of antioxidants to scavenge free radicals and chelate prooxidative transition metals are the most common methods used to reduce lipid oxidation (Decker, 2002; Frankel, 2005), since the complexity of both food and skin care products requires both these properties to maintain oxidative stability. Therefore multifunctional antioxidants are wanted. Polyphenolic compounds are often multi-functional antioxidants exhibiting both radical scavenging and metal chelating activity, due to their structure and location of OH-groups (will be further discussed in section 4.4.4). Therefore, naturally occurring polyphenolic compounds have great potential as natural antioxidants in both food and skin care o/w emulsions.

4.2. Antioxidant efficacy theories and their partitioning in emulsion systems

In multiphase emulsion systems, the antioxidant may partition into at least three different phases: the aqueous phase, the oil phase and the oil-water interface, depending on the polarity of the antioxidants. Fig. 4.1 shows the primary location of the antioxidants according to their polarity in emulsions.
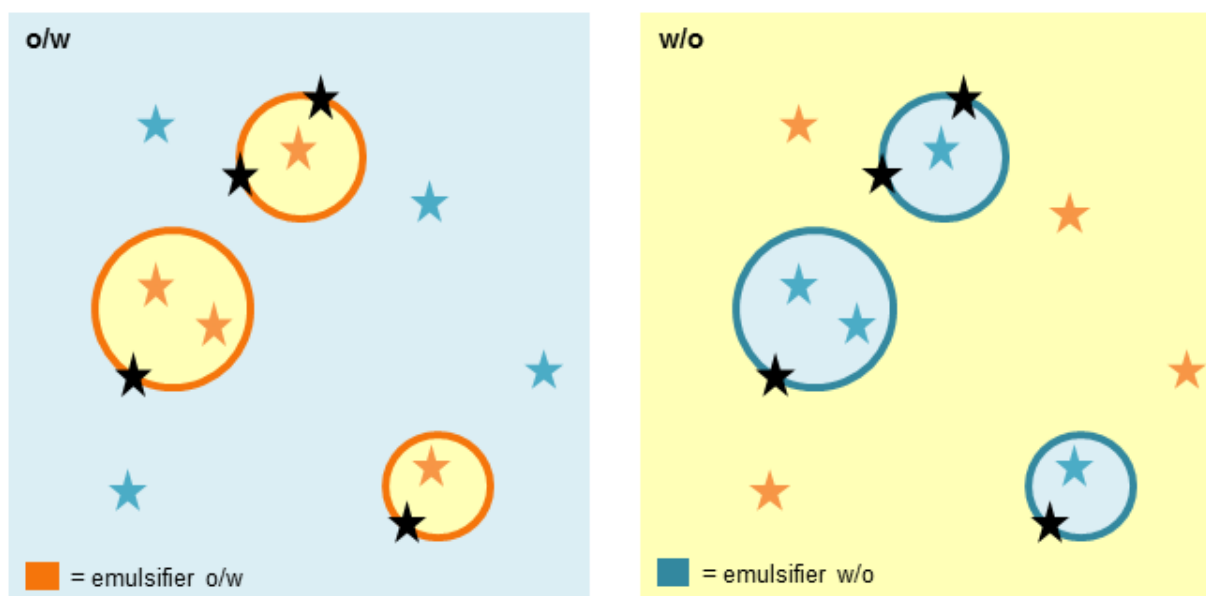

Fig. 4.1. Simplified structure of oil-in-water (o/w) and water-in-oil (w/o) emulsions. The location of antioxidants in these emulsions is indicated according to their polarity. Amphiphilic antioxidants ★ Lipophilic antioxidants ★ and Hydrophilic antioxidants ★

Antioxidant efficiency has been measured and compared in different systems, e.g. bulk oil, o/w emulsions and w/o emulsions (Frankel et al., 1994; Frankel et al., 1996; Huang et al., 1996a; Huang et al., 1996b; Schwarz et al., 2000). The efficiency of the antioxidants has been found to be dependent on the systems in which their effect is evaluated. According to the polar-paradox hypothesis, suggested more than twenty years ago, the polarity of the antioxidants determines their antioxidant efficacy since polar antioxidants where suggested to be more efficient in non-polar media such as oil, whereas non-polar antioxidants would be more efficient in more polar systems. Thus, the affinity of the antioxidants towards the different phases determines the efficacy of the antioxidants. Moreover, lipophilic antioxidants are more efficient in emulsions due to their ability to orient themselves closer to the oil-water interface where the lipid oxidation is initiated, whereas hydrophilic antioxidants are diluted in the water phase and therefore are less efficient (Frankel et al., 1994).

However, the polar paradox theory has recently faced challenges as further studies revealed inconsistent results, some of which are not explained exclusively by this theory (Sørensen et al., 2008; Lauguerre et al., 2009). The action of antioxidants is possibly governed by more complex phenomena than polarity, and other factors must be taken into account to explain the efficacy. Lauguerre et al. (2009) observed a non-linear behaviour of lipophilic alkyl esters of a phenolic antioxidant in emulsion systems, as the antioxidant efficacy increased with increasing alkyl chain length. This was observed until a threshold was reached, and a drastic decrease in antioxidant efficacy was observed as the alkyl chain length continued increasing. The increased efficacy of phenolic compounds with increased alkyl chain length was related to the location of the phenolics, as these got more and more associated with the oil-water interphase due to increased lipophilization. Above the "critical chain length" Lauguerre et al. (2009) suggested that micellation of the antioxidants occurred in the water phase, which resulted in decreased antioxidant efficacy. This nonlinear phenomenon of polarity is referred to as the cut-off effect, and shows that the partitioning of antioxidants in emulsions is important. However their antioxidant efficacy in e.g. food systems can be influenced by other factors than the polarity, such as type of emulsifiers and concentration of emulsifiers, and possibly viscosity (Alemán et al., 2015).

4.3. Antioxidant strategies to hinder lipid oxidation oil-in-water emulsions

The antioxidant strategy differs in different products depending on their physical and chemical compositions. Hence, it has been shown that it is not always possible to extrapolate results of an antioxidant in one product to another, since the antioxidant can be efficient in one product and not in another (Nielsen & Jacobsen, 2009; Sørensen et al., 2015; Alemán et al., 2015). In the following the antioxidant strategies in food and skin care products similar to those used in present PhD

work will be discussed to understand what type of antioxidant mechanisms are important in these types of products.

4.3.1. Antioxidant application in the industry

Antioxidants are widely used by the industry to enhance oxidative stability of lipid rich products. Synthetic antioxidants, e.g. BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), are widely applied by the industry. BHA and BHT are both primary antioxidants and efficient radical scavengers. The synthetic antioxidants are typically cheaper and can be easier to process than natural antioxidants. However, restrictions in the use of synthetic antioxidants have been enforced because of their health risks and toxicity (Branen, 1975; Linderschmidt et al., 1986). Hence there is a significant interest in and demand for replacing synthetic antioxidants with natural plant-based alternatives, not only due to safety issues but also due to increased consumer awareness and interest in natural products and the possible health benefits of natural antioxidants (Halliwell, 1996).

Tocopherols is an example of a natural antioxidant applied by the industry, i.e. the food industry (Löliger, 1991). They act as primary antioxidants by radical scavenging and thereby directly inactivate free radicals. Tocopherol has shown prooxidant activity when used in high concentrations of α-tocopherol in for example edible oils (Huang et al., 1994). Hence, the antioxidant activity of tocopherol is highly concentration dependent and α-tocopherol has been shown to be an efficient antioxidant in 10% o/w model systems within a range from 65-129 ppm (Frankel et al., 1994; Huang et al., 1996a).

Another natural antioxidant used by the food industry is ascorbic acid. Whereas tocopherol is hydrophobic, ascorbic acid is hydrophilic. Ascorbic acid is known to have multi-functional antioxidant activity since it can act as radical scavenger of hydrophilic radicals, oxygen scavenger, and donate an electron to reactive free radicals (reducing properties). Furthermore, ascorbic acid can act synergistically with tocopherol by regenerating tocopheroxyl radicals (Niki, 1991; Mäkinen et al., 2001). However, the reducing properties of ascorbic acid can also have a negative effect on lipid oxidation since it can reduce Fe^{3+} to Fe^{2+}, which is highly reactive and reacts with already existing lipid hydroperoxides. Hence, Jacobsen et al. (1999a) have shown that the reducing properties of ascorbic acid on iron resulted in prooxidative effects in FO-enriched mayonnaise.

4.3.2. Antioxidant strategies in fish-oil-enriched milk

EDTA has been found to be an efficient antioxidant in milk enriched with unsaturated oils (Let et al., 2003; Timm-Heinrich et al., 2003). Timm-Heinrich et al. (2003) evaluated EDTA and gallic acid in milk drinks with sunflower oil and found that EDTA was a strong antioxidant, while gallic acid did

not exert a distinct antioxidative effect. Furthermore, EDTA reduced lipid oxidation in FO-enriched milk (Let et al., 2003). Let et al. (2005) evaluated the oxidative stability of FO-enriched milk by either addition of antioxidants (e.g. EDTA and ascorbyl palmitate) or rapeseed oils using the naturally occurring antioxidant compounds, i.e. phenolic compounds and tocopherols, in the oils to stabilise the enriched milk. They found no effect of EDTA, and related this lack of activity to the quality of the FO, since factors like low peroxide value (PV) (high quality oil) made the oil stable in the milk and therefore less susceptible to trace-metal-mediated degradation of hydroperoxides. Instead they observed antioxidant activity of primary antioxidants (ascorbyl palmitate), which almost completely retarded oxidation in the FO-enriched milk. The addition of rapeseed oil containing natural antioxidants to FO was equally efficient in inhibiting oxidation in the milk.

4.3.3. Antioxidant strategies in fish-oil-enriched mayonnaise

Studies have shown that tocopherols are located in the oil phase of mayonnaise, which then according to the theory, should be efficient as antioxidants (Jacobsen et al., 1999b). However, only poor antioxidant activity of tocopherol has been found in FO-enriched mayonnaise (Jacobsen et al., 2000; Jacobsen et al., 2001a). The polarity and thereby the partitioning properties of the antioxidants thus plays a minor role in this system. The differences in antioxidant efficiency between simple emulsion systems and more complex systems can be due to the oil content (10% vs 80%) and the use of more complex emulsifiers, e.g. egg yolk in mayonnaise. Egg yolk has high iron content (40 µg/g), which can accelerate the oxidation as described in Chapter 3. The synthetic polar antioxidant EDTA has been found to be the most efficient inhibitor of metal-catalysed oxidation in FO-enriched mayonnaise (Jacobsen et al., 2001b; Nielsen et al., 2004). This is due to its ability to prevent metal-catalysed degradation of peroxides by metal chelation.

4.3.4. Antioxidant strategies in fish-oil-enriched granola bars

In other systems like FO-enriched energy bars with FO added as 70% FO/w preemulsion, Nielsen & Jacobsen (2009) found that the EDTA, caffeic acid and ascobyl palmitate worked as prooxidants, irrespective of the concentration added. On the other hand, addition of γ-tocopherol to similar energy bars was efficient in concentrations above 440 µg γ-tocopherol/g FO (Horn et al., 2009). This was probably due to the location of γ- tocopherol at the oil-water interface or inside the oil droplets after emulsification, where it could work as H-donor and thereby scavenge free radicals.

Barden et al. (2015a) studied the influence of iron on lipid oxidation in low-moisture crackers added soybean oil and the antioxidant efficacy of EDTA in this system. They found that metal chelating activity was less important and suggested that free radical scavengers would be the best solution to stabilize lipids in this type of foods since they also found no effect of EDTA. In an additional

study by Barden et al. (2015b) the antioxidant efficacy in low-moisture crackers added neat oil was found to increase with increased hydrophobicity of esterified phenolic compounds (Barden et al., 2015b). It was suggested that the water in such low-moisture foods is associated with starch or protein and is not dispersed as droplets, giving fewer water-oil interactions and thereby fewer interactions between water-soluble prooxidants like iron and the lipids. Based on these findings it can be assumed that radical scavenging activity is the most important antioxidant property for efficient lipid oxidation inhibition in foods similar to crackers, i.e. FO-enriched granola bars.

In addition to the use of antioxidants, the oxidative stability of emulsified oil can also be increased by controlling emulsifier type, location, and concentration (Grigoriev and Miller, 2009). Creating thick interfacial layers around emulsion droplet membranes that hinder interactions between water soluble prooxidants and lipids inside the emulsion droplet may also inhibit lipid oxidation. For example, when oil-in-water emulsion droplets are surrounded by cationic emulsifiers, prooxidant metals are repelled and lipid oxidation rates decrease (Klinkesorn 2005, Jiménez-Martín 2015; Shaw 2007). In a study by Klinkesorn (2005), the lipid oxidation during storage was slower in the secondary emulsions stabilized by lecithin-chitosan as compared to primary emulsions produced by lecithin alone, oxidation markers were still observed to increase over time. Therefore, the presence of antioxidants is needed to further decrease the lipid oxidation.

4.3.5. Antioxidant strategies in skin care products

Unlike antioxidant application in foods the use of antioxidants in skin care products such as facial cream, are due to their multi-functional activities, such as anti-aging and anti-inflammatory effects (Dutot et al., 2012). The skin is the first line of defences, which protects us against environmental, chemical and physical factors. Chronic exposure to factors like ultraviolet (UV) radiation, harmful bacterial growth or chemical/metal contact causes damage to the skin leading to chronic irritation or premature aging. A factor like UV radiation causes excitation of electrons and thereby generation of photochemical reactions resulting in lipid oxidation of the skin. This leads to an inflammatory condition in the skin causing membrane damage, decreased membrane fluidity, and in worst-case skin cancer. Hence, application of efficient photoprotective agents in facial cream is important to maintain healthy skin. Kaur and Saraf (2012) tested extracts from turmeric (*Curcuma longa*) and pomegranate (*Punica granatum*) rich in polyphenolic compounds for photoprotection in cream. They found that due to this high polyphenolic content in the herbal extracts the photoprotection of the creams was increased.

Besides protection of the skin, the composition of lipids in the skin care product determines the functionality of skin care products, like facial cream. Lipid oxidation of fatty acids like linoleic acid, will lead to development of undesirable odours in the product, and decrease the effect of this fatty

acid in the cream (Zielinska and Nowak, 2014). Therefore, protection of PUFAs by addition of antioxidants to facial cream is essential.

4.3.6. Future antioxidant strategies

Due to the fact that some of the available natural antioxidants, such as tocopherol and ascorbic acid, do not have a satisfying efficacy in most FO-enriched foods, the efficacy of natural mono-compound antioxidants can be questioned. A search for multi-functional additives with antioxidant activity, e.g. extracts from plants rich in different phenolic compounds, is currently ongoing (review from Patel, 2015). Antioxidant activity of extracts from oregano (*Origanum vulgare*), rosemary (*Rosmarinus officinalis*) and green tea has been evaluated. Oregano extracts evaluated in n-3 rich food systems (from either linseed or FO), where found to reduce lipid oxidation in a dairy beverage (Boroski et al., 2012). Extracts from rosemary (750 mg/kg) or green tea (650 mg/kg) was able to partially mask the fishy taste in surimi when prepared with FO concentrates. However, a weak prooxidative effect was found for both extracts in surimi when surimi was fortified with fish oil (Pérez-Mateos et al., 2006). More multi-functional extracts from natural sources need to be evaluated.

4.4. Natural antioxidants from brown algae

Seaweeds contain a wide range of bioactive substances like tocopherols, pigments, e.g. carotenoids and chlorophylls, sulphated polysaccharides, peptides, amino acids, metals, and polyphenolic compounds (Holdt and Kraan, 2011). The bioactive compounds all exhibit some degree of antioxidant, prooxidant or synergistic activity. The following paragraph will discuss the properties of tocopherols, carotenoids, chlorophylls, polyphenolic compounds and metals found in *F. vesiculosus* and their influence on the antioxidant efficacy of *F. vesiculosus* extracts.

4.4.1. Tocopherols

Tocopherols are highly potent antioxidants and widely used by the food industry due to their efficient radical scavenging activity. Tocopherols can be found in four isomers: α-, β-, γ-, δ-tocopherol (Fig. 4.3). The antioxidant activity order of the four tocopherol isomers in bulk oil is: δ > γ > β > α (Decker, 2002).

Tocopherols, mainly α-tocopherol, are found in *Fucus* sp. in small amounts, 38-73 mg/kg dw depending on the season (Jensen, 1969). However, their ability to work as antioxidants in the oil phase or at the oil-water interphase or emulsions might contribute significantly to the antioxidant activity of *F. vesiculosus* extracts in foods and skin care products.
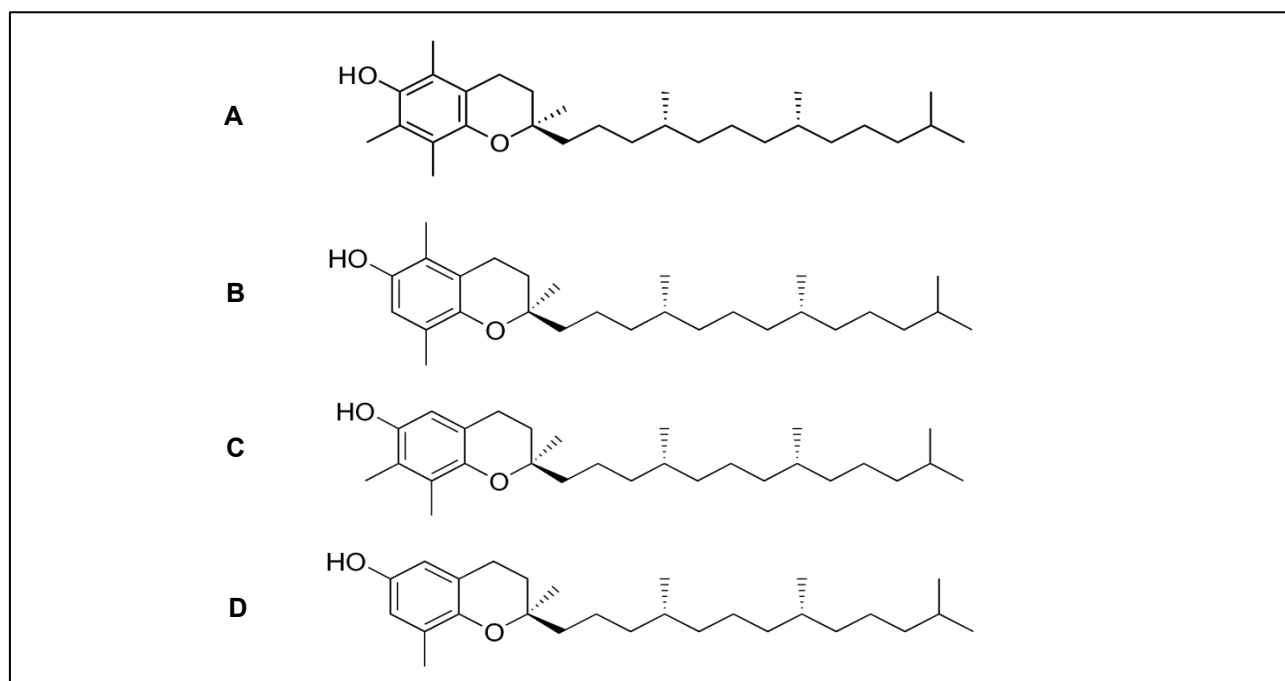
Fig. 4.3. Structure of four tocopherol isomers (α- (A), β- (B), γ- (C), and δ-tocopherol (D))

4.4.2. Pigments

Pigments found in brown algae such as carotenoids and chlorophylls can exhibit antioxidant activity. Furthermore, these pigments can work synergistically with tocopherol (Le Tutour et al., 1998).

Carotenoids are lipophilic molecules, which play a major role in the protection of plants against photooxidative processes. They are efficient antioxidants scavenging singlet oxygen and peroxyl radicals, and their synergistic interactions with other antioxidants can enhance the antioxidant efficacy of mixtures compared with the single compound (Truscott, 1990; Böhm et al., 1997; Young and Lowe, 2001).

The unique structure of carotenoids determines their potential biological functions and actions (Britton, 1995). Most carotenoids can be derived from a 40-carbon basal structure, which includes a system of conjugated double bonds. The central chain may carry cyclic end-groups, which can be substituted with oxygen-containing functional groups. Based on their composition, carotenoids are divided in two classes, carotenes containing only carbon and H-atoms, and oxo-carotenoids (xanthophylls), which carry at least one oxygen atom. The pattern of conjugated double bonds in the polyene backbone of carotenoids determines their light absorbing properties and influences the antioxidant activity of carotenoids. Moreover, just like ascorbic acid, β-carotene regenerates tocopherol from the tocopheroxyl radical, providing a synergistic effect in systems where a combination of these is present (Böhm et al., 1997).
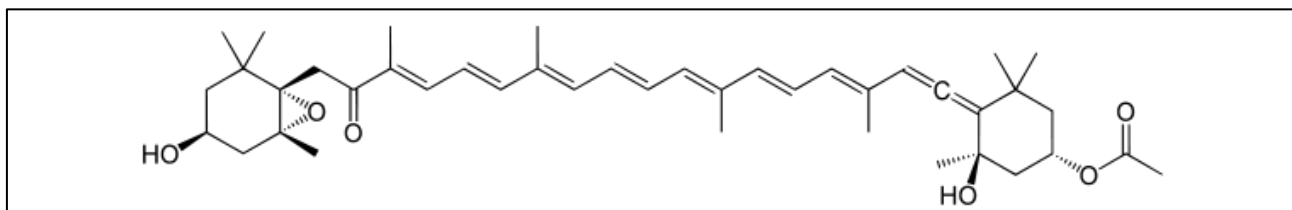
Fig. 4.2. Structure of Fucoxanthin

β-carotene, violaxanthin and fucoxanthin are all present in brown seaweed species (Haugan and Liaaen-Jensen, 1994). The most abundant carotenoid is fucoxanthin, a xanthophyll, which has a very intense brown color. Fucoxanthin has a unique structure including an allenic bond (=C=) and a 5,6-monoepoxide in the molecule (Fig. 4.2) (Nomaru et al., 1997; Yan et al., 1999; Dembitsky & Maoka, 2007). Nygård and Ekelund (2006) found that *F. vesiculosus* contained 0.2 µg/mg dw β-carotene and 1 µg/mg dw fucoxanthin. Xanthophylls are efficient quenchers of singlet oxygen. This antioxidant activity is relevant when photooxidation (oxidation initiated by light) occurs. Under other conditions where singlet oxygen is not formed, these carotenoids can work as scavengers of peroxyl radicals (Nomura et al., 1997; Le Tutour et al., 1998). Moreover, carotenoids can act as prooxidants under high oxygen pressure (Burton & Ingold, 1984). The brown colour of fucoxanthin is efficient to absorb light; hence fucoxanthin participates in the photosynthesis as an accessory pigment (Vijayan & Somayajula, 2014).

Another major pigment group found in brown algae is chlorophyll. Nygård and Ekelund (2006) found that *F. vesiculosus* contained 5 µg/mg dw chlorophyll a. Chlorophylls as well as oxidised or breakdown products hereof can have severe pro-oxidative effects, since they can work as sensitizers in photooxidation and the formation of singlet oxygen (Usuki et al., 1984; Andersen & Skibsted, 2010). Hence, the prooxidant activity of chlorophylls requires photooxidation conditions, such as light. However, chlorophylls can also provide protection by preventing autoxidation of vegetable edible oils stored in the dark due to a H-donating mechanism breaking the radical chain reactions (Endo et al., 1985).

4.4.3. Metals

Both minerals and heavy metals are found in seaweed. Heavy metals such as Zn, Cu, Pb and Cd are present in the environment and may be accumulated in the seaweed (Boubonari et al., 2008). However, some seaweed species are more efficient in accumulating such compounds than others. Hence, the presence of undesired metals should be taken into account and tested if seaweed species are to be used as ingredients for the food and cosmetic industry, since such metals might be concentrated during extraction of antioxidant compounds from seaweed.

Fucus vesiculosus contains metals, i.e. copper (Cu) and iron (Fe) (van Netten et al., 2000). Van Netten et al. (2000) found that Fe was the most abundant metal in *F. vesiculosus* (520 μg Fe/g dw), while Cu was only found in trace amount (1.4 μg Cu/g dw). As described in Chapter 3, transition metals like Cu and Fe stimulate lipid oxidation and are very important initiators in some food products such as mayonnaise as they can act as prooxidants. In order though for Fe and Cu to participate in lipid oxidation they need to be in an active form, i.e. metal ions, and not in transition state or bound to e.g. proteins or polysaccharides.

4.4.4. Phenolic compounds

Naturally occurring phenolic compounds found in seaweeds comprise one or more hydroxyl group (–OH), which bonds directly to an aromatic hydrocarbon group. The antioxidant activity of phenolic compounds can be related to the structure, since they are able to scavenge free radicals and chelate metal ions due to the presence of hydroxyl groups. In order for phenolic compounds to chelate metal ions, two neighbouring OH groups (*o*-diphenol) are required in the structure (Chimi et al., 1991; Andjelkovic et al., 2006). The ability to react with radicals correlates with the number of phenolic rings and catecholic structures (*o*-diphenol) in the molecule (Capitani et al., 2009).

Brown algae contain polyphenolic compounds called phlorotannins, which are likely to be bound to proteins and carbohydrates (Stern et al., 1996; Ragan and Glombitza, 1986). Due to the complex structure of phlorotannins, they are efficient antioxidants and have potential as multi-functional natural antioxidants. The occurrence, structure and antioxidant efficacy of phlorotannins will be further discussed in Chapter 5.

4.5.   References

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Chapter 5: Phlorotannins as novel natural antioxidants

Phlorotannins are recognised for their good antioxidant activity and are therefore of interest in the search for new natural antioxidants to be applied by the industry. This chapter will focus on the structure, biosynthesis and role of phlorotannins and their ability to work as antioxidants. Furthermore, methods for extracting and characterising phlorotannins will be discussed.

5.1. Phlorotannins; structure, synthesis, role and seasonal variation

Phlorotannins are oligo- or polymers of phloroglucinol (1,3,5-trihydroxybenzene) (Fig. 5.1-a) and may contribute up to 25% of the dw in brown algal species (Ragan and Glombitza, 1986). Phlorotannins are water-soluble and occur in physodes, i.e. membrane-bound cytoplasmic vesicles, where they perform different biological tasks (Baardseth, 1958).

5.1.1. Structure and classification of phlorotannins

Naturally occurring phlorotannins can be classified according to their linkage of phloroglucinol units (PGU) (Ragan & Glombitza, 1986; Targett & Arnold, 1998). The molecular weight of phlorotannins varies from 0.126 to 650 kDa, although the most common observed range is from 10 to 100 kDa (Steevensz et al., 2012). According to Martínez & Castañeda (2013) there are three main groups of phlorotannins: 1) fucols, 2) phloroethols and 3) fucophloroethols (Fig. 5.1). By increasing the number of PGUs, the structural diversity and complexity increases, as the PGU linkages can be linear or branched or both.

Fucols

Fucols are phlorotannin polymers in which the PGUs are connected only by C-C (phenyl linkage) bonds in meta position (Fig. 5.1-b). Fucols identified from *F. vesiculosus* can be linear or branched structures, e.g. tetrafucol A and tetrafucol B (Truus et al., 2004).

Phloroethols

The PGUs in phloroethols are linked only by C-O-C (aryl-ether) bonds (Fig. 5.1-c). The linear phloroethols may have ortho-, meta- or para-oriented biphenyl ether bridges. A subgroup of phlorethols is eckol (Fig. 5.1-e) and carmalols, which structurally include a 1,4-dibenzodioxin linkage (Singh & Bharate, 2006).

Fucophloroethols

Fucophloroethols are a mixture of both biaryl and aryl-ether bonds (Fig. 5.1-d) allowing a variety of compounds in linear, branched and heterocyclic fashions. Trifucodiphloroethol A and

trifucotriphloroethol A are examples of two branched fucophloroethols, which have been identified from *F. vesicolusus* (Parys et al., 2010).
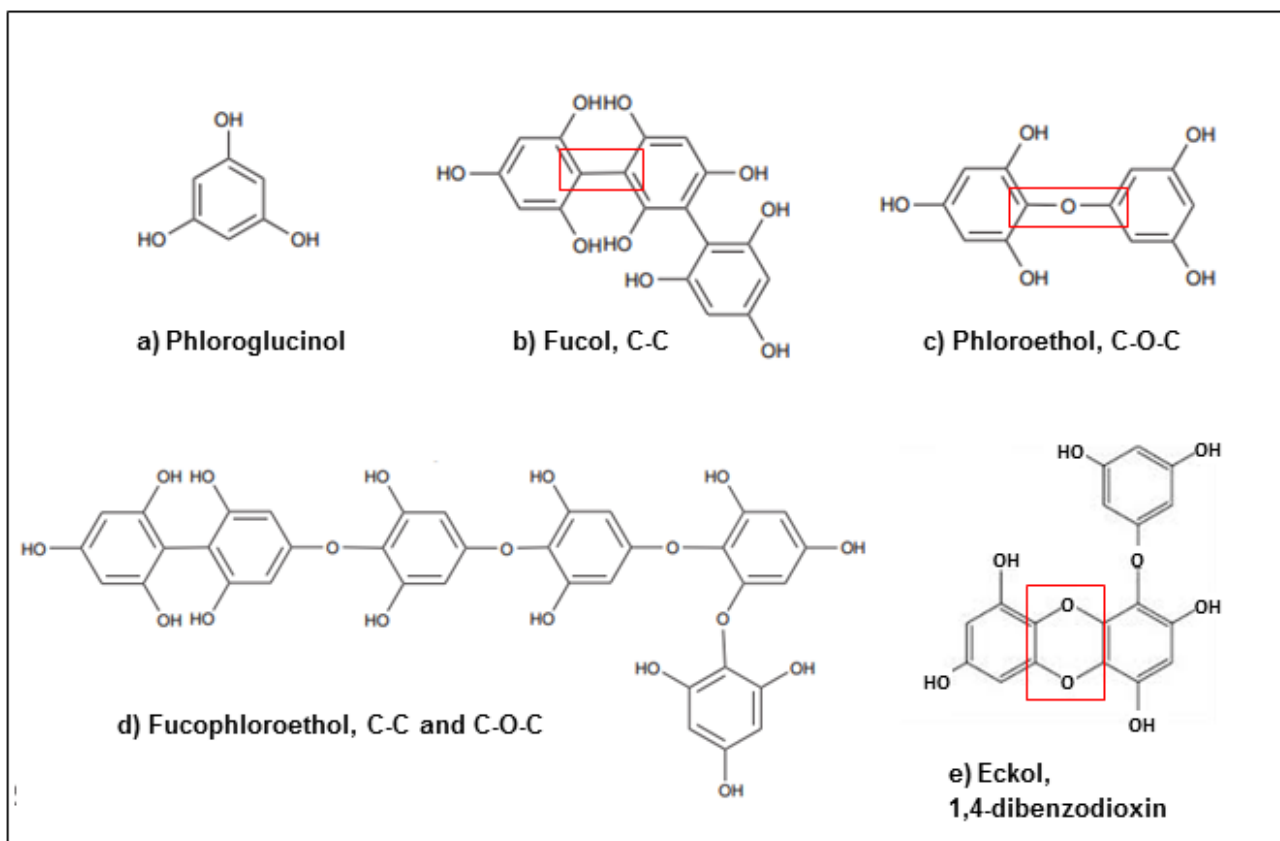

Fig 5.1. Chemical structure of phloroglucinol (a) and main classes of phlorotannins derived from brown algae: (b) fucol; (c) phloroethol, and (d) fucophloroethol, and the subgroup (e) eckol. The different linkages are marked (Adapted from Martínez & Castañeda, 2013)

5.1.2. Synthesis of phlorotannins

In the early steps of phlorotannins biosynthesis, two molecules of acetyl co-enzyme A (CoA) are converted into malonyl CoA through addition of CO_2 as part of the acetetyl-malonyl pathway (or the polyketide pathway) (Arnold and Targett, 2002). In Fig. 5.2 the conversion of malonyl-CoA into the methylene is shown. This methylene is highly reactive and assists the polymerisation process to occur without the need for a high energy investment (Waterman and Mole, 1994).
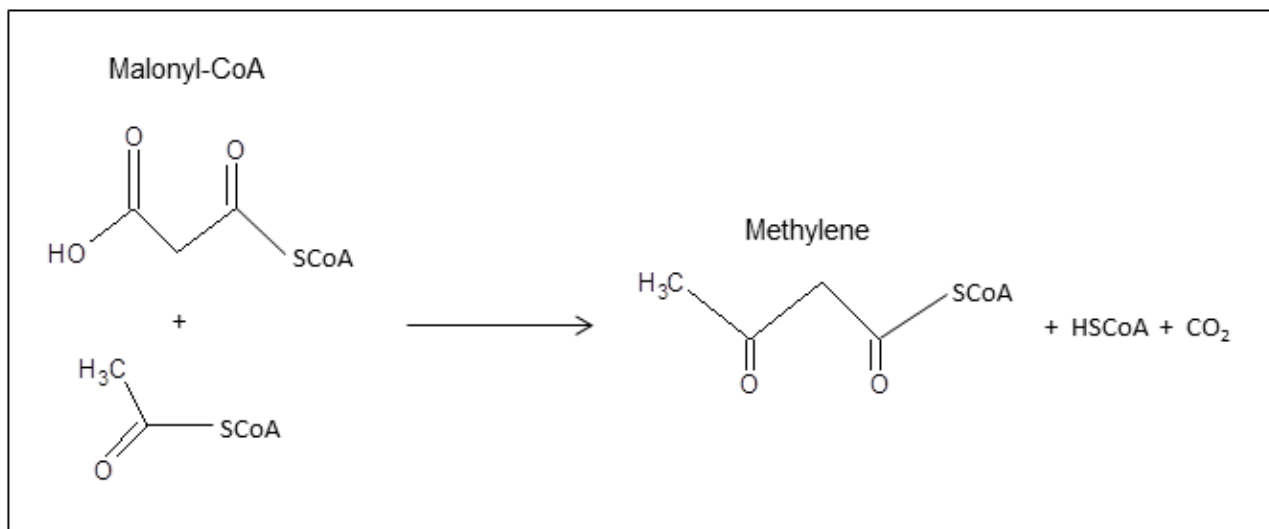
Fig. 5.2. Formation of highly reactive methylene (modified after Waterman and Mole, 1994)

Methylene compounds polymerise and the result is a polyketide chain with an acid group. This chain forms a hexacyclic ring system by eliminating water, forming an unstable triketide which is further transformed to the more stable aromatic form, phloroglucinol, consisting of three phenolic hydroxyl groups (Fig. 5.3) (Waterman and Mole, 1994). Further mechanisms of the biosynthetic pathway are unknown.
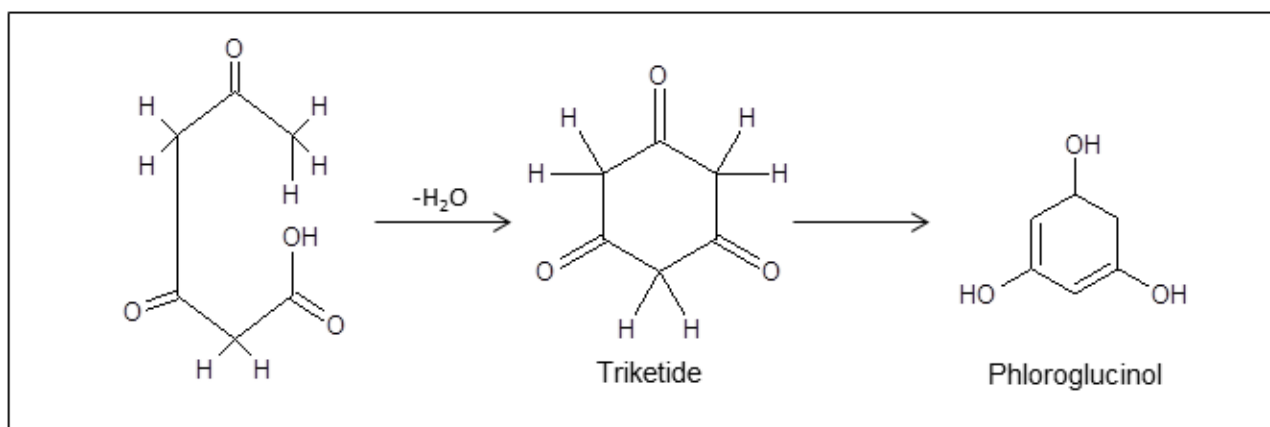

Fig. 5.3. Cyclisation of a triketide chain to form phloroglucinol (modified after Waterman and Mole, 1994)

5.1.3. Role of phlorotannins in brown algae

There are multiple roles of phlorotannins, e.g. strengthening of the cell wall, an ability to bind metal ions and absorbtion of UV radiation to protect the seaweed from UV damage. They can also act as antibacterial agents, fouling inhibitors and herbivory deterrents (Pavia et al., 1997; Pavia & Brock, 2000; Hemmi et al., 2004). Accumulation of phlorotannins has been found around wounds caused by herbivores suggesting a wound healing role of phlorotannins (Pavia & Brock, 2000; Lüder &

Clayton, 2004). The concentration of phlorotannins has also been found to vary in response to environmental parameters such as salinity, available nutrients, light and UV irradiation (Baardseth, 1958; Targett & Arnold, 1998; Hemmi et al., 2004).

5.1.4. Seasonal variation of phlorotannin content

Seasonal variation in phlorotannin levels (from 3 to 10% of the dw) has been reported in *F. vesiculosus* collected from shores in Brittany (France) and western Scotland. In cold water areas the phenolic content is typically high in summer and early autumn whereas lower levels have been observed during winter (Connan et al., 2004; Parys et al., 2009). The high levels of phlorotannins in brown algae during summer are due to the UV-radiation response. Hence, to obtain *F. vesiculosus* extracts rich in phlorotannins, the harvesting time is very important and should be considered in a potential production of natural antioxidants derived from *F. vesiculosus*.

5.2. Extraction of phlorotannins

It is crucial to develop an extraction method which can generate high yields of phlorotannins in order to obtain antioxidant efficient extracts, which can subsequently be applied to food and skin care products.

Solid-liquid extraction (SLE) methods using solvents is the most frequently applied extraction method. However, in recent decades, several highly efficient and environmental friendly techniques have been developed for extraction of natural antioxidants from plant material, including supercritical water extraction (SWE) and pressurised liquid extraction (PLE) (Plaza et al., 2010; Plaza et al, 2013). The advantage of these methods is first of all the reduction in use of toxic and hazardous solvent. Furthermore, these techniques have high selectivity, high extraction efficacy, short extraction time, they can be automated, and retain the sample in an oxygen and light-free environment in contrast to traditional SLE.

In this PhD project SLE with solvents with different polarity was applied. Furthermore, PLE was applied to investigate the possibility of extracting phlorotannins using "green" extraction techniques. In the following the fundamentals of the two applied extraction techniques are explained.

5.2.1. Solid-liquid extraction

SLE with organic solvents is typically applied when extracting phenolic compounds from plant material and algae. The yield of phenolic compounds depends on their solubility in the given solvent. In earlier studies it was thus found that water was inferior to polar organic solvents in extracting polyphenolic compounds from *F. vesiculosus* (Wang et al., 2009; Farvin & Jacobsen,

2013). Hence, the effective extractants recommended are ethanol and acetone (Waterman & Mole, 1994) or aqueous solutions of these (Koivikko et al., 2005; Wang et al., 2009; Farvin & Jacobsen, 2013). Yotsu-Yamashita et al. (2013) produced a crude polyphenol powder from brown alga *Ecklonia kurome* by extraction with ethanol:water (70:30, v/v) with a polyphenol content of 70% (w/w) of the total phenolic content (TPC). The effectiveness of ethanol might be due to the fact that ethanol precipitates most of the proteins and leaves some of the reversibly bonded phenolic compounds into the solution. Extraction with 70% acetone has been found to have high phlorotannin extraction efficacy due to a limited formation of protein-polyphenol complexes or even break down of H-bond in the complexes and thereby release of phlorotannins from protein during extraction (Kallithraka et al., 1995).

5.2.2. Green extraction methods

PLE has been found to successfully extract phenolic compounds from natural samples (Plaza et al., 2013). PLE is based on the use of a combination of temperature and pressure where solvents are maintained in their liquid state during the whole extraction process. Tierney et al. (2012) evaluated the extraction of phenolic compounds from brown algae by PLE and traditional SLE. They found that a high TPC was achieved by PLE when using acetone:water (80:20). However, when using "green" solvent combinations, e.g. ethanol:water (80:20) or water, to extract phenolic compounds from *Fucus spiralis*, SLE generated higher TPC compared to PLE.

5.3.   Identification and characterisation of phlorotannins

The TPC in brown seaweed extracts is usually determined by the Folin-Ciocalteu assay (Singleton & Rossi, 1965; Wang et al., 2009; Farvin & Jacobsen, 2013). For further identification and characterisation of phlorotannins advanced detection methods should be applied.

5.3.1. Quantification of phlorotannins

The principle of the Folin-Ciocalteu assay involves oxidation of phenolic rings by phospotungstic and phosphomolybdic acids, resulting in the formation of a blue complex and molybdene oxides which can then be detected spectrophotometrically between 725 and 765 nm. The total phenolic content can then be expressed as an equivalent quantity of typically gallic acid or phloroglucinol. However, it is questionable whether this assay is suitable as an absolute measurement of the amount of phenolic compounds, since a range of non-phenolic substances can interfere with the assay and result in an over-estimation of TPC (Singleton et al., 1999; Ikawa et al., 2003). Quantitative nuclear magnetic resonance (qNMR) is an alternative method to quantify natural compounds (Parys et al., 2007; Jégou et al., 2015). Parys et al. (2007) proved though that both

Folin-Ciocalteu assay and qHNMR were most reliable and precise methods for determining the quantity of phlorotannins in *F. vesiculosus*.

5.3.2. Advanced identification and characterisation of phlorotannins

The polar nature and high solubility, in e.g. polar solvents, of phlorotannins makes liquid chromatography suitable for separation of these compounds. Advanced detection methods such as tandem mass spectrometry (MS^2) and NMR can be combined with the e.g. liquid chromatography (LC) or HPLC for a rapid structural identification of phlorotannins. Sample preparation is important to improve the separation of phlorotannins and thereby also the detection of these. In the following section the best way for separation and identification of phlorotannins will be discussed.

Sample preparation

Sample preparation is important in the analysis of phlorotannins from algal material. Phlorotannins possess chemical properties that enable their extraction and purification, allowing purified extracts or fractions to be obtained. Such purification improves the separation of the phlorotannins when applying liquid chromatography (LC) or high performance liquid chromatography (HPLC) as separation technique, as it removes interfering compounds.

Simple purification of crude extracts using liquid-liquid partitioning (LLP) by highly polar solvents can be one method of sample preparation. Wang et al. (2012) purified an 80% (v/v) ethanol *F. vesiculosus* extract by LLP with, e.g. ethyl acetate. Further purification of the ethyl acetate fraction was performed using column chromatography. This technique yields fractions depending on the analytes affinity to the stationary phase.

Separation and detection

Chromatographic separation of analytes by differential adsorption between a stationary phase and a mobile phase combined with a detector, typically an ultraviolet–visible (UV/Vis) diode array detector (DAD) can be used in detection of phlorotannins. Koivikko et al. (2007) evaluated separation of phlorotannins by either reverse phase (RP) or normal phase (NP) HPLC. They were able to separate phlorotannins by NP-HPLC, and hypothesised based on the method that phlorotannins emerged in the order of the degree of polymerisation, the shorter oligomers first followed by the longer polymers, i.e. phlorethols < fucophlorethols < fucols, based on the number of free hydroxyl groups.

Structural elucidation of phlorotannins

Structural elucidation of phlorotannins is a rather complex task, even more so as no reference compounds are commercially available, only the monomer, phloroglucinol.
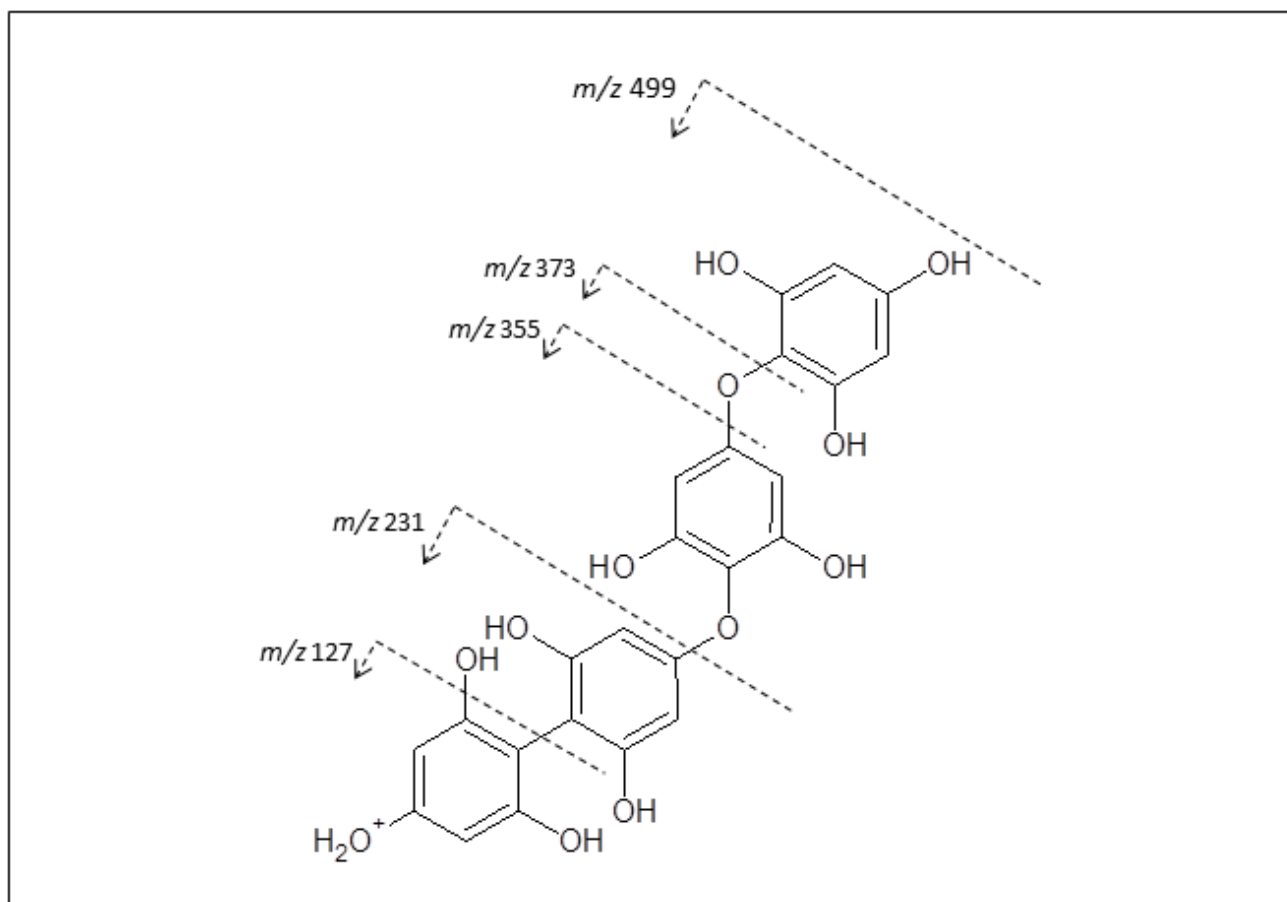
Fig. 5.4. Structure of a possible phlorotannin isomer (e.g. fucodiphloroethol A, C24H18O12, FW: 498.3925) with protonated molecular ions ([M+H]⁺) at m/z 499 and its proposed fragmentation pathways (adapted from Sandsdalen et al., 2003; Wang et al. 2012; Ferreres et al., 2012)

Advanced detection methods like tandem MS can be used for phlorotannin elucidation. Obtained MS data can with advantage be compared with theoretical mono-isotopic masses corresponding to phlorotannins found in literature. Ferreres et al. (2012) used this approach as they applied HPLC-DAD-ESI-MS[2] to identify phlorotannins in purified extracts from four brown algae. They used known isotopic masses of phlorotannins to conduct extracted ion chromatograms (EIC) of protonated molecular ions ([M+H]⁺) of these (e.g. eckol (*m/z* 373), fucophloroethol (*m/z* 375), fucodiphloroethol (*m/z* 499), phlorofucofuroeckol (*m/z* 603), fucotriphloroethol (*m/z* 623), dieckol (*m/z* 743), and fucophloroethols with six (*m/z* 747), seven (*m/z* 871) and eight (*m/z* 995) PGUs). This resulted in EICs with well-defined and abundant ions of 12 possible phlorotannins. The MS study of the ions allowed detection of e.g. two isomers with protonated molecular ions ([M+H]⁺) at *m/z* 499.4. Their MS[2] fragmentation patterns was similar and characteristic for fragmentation of phlorotannins, with loss of a water molecule (*m/z* 481, -18), one PGU (*m/z* 373, -126), as well as the protonated molecular ion of phloroglucinol (*m/z* 127), the base peak being the ion resulting from the loss of one PGU and water (*m/z* 355, -126, -18). They tentatively identified the ions as two

phlorotannin tetramers, possibly fucudiphloroethol isomers. The fragmentation pathways for fucudiphloroethol are suggested in Fig. 5.4. It is most likely that the fragmentation of fucodiphloroethol isomers will start with the aryl-ether bindings since these bindings are more liable to be fragmented than the phenyl linkage.
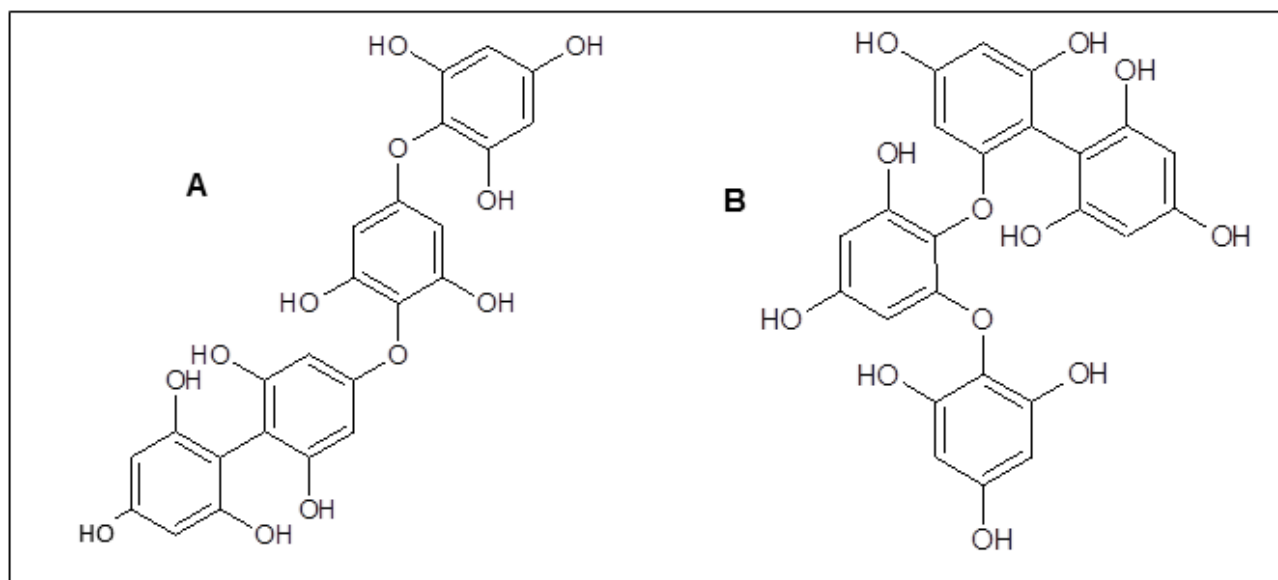

Fig. 5.5. Structure two phlorotannin tetramers, A: fucodiphloroethol A and B: fucodiphloroethol G (Sandsdalen et al., 2003; Kim et al., 2014)

Such isomers, with the same molecular weight, can occur, depending on the linkage between the four PGUs and thereby the branching of the compound, e.g. fucodiphloroethol A and fucodiphloroethol G (Fig. 5.5) (Steevensz et al., 2012).

A high number of isomers have been detected for individual deprotonated molecular ions in brown algae due to the significant variation in branching positions between PGUs in the phlorotannins (Heffernan et al., 2015). Heffernan et al. (2015) primarily detected low molecular weight phlorotannins in *F. vesiculosus*, ranging from 3 to 16 PGUs, e.g. [M-H]⁻ *m/z* 497 (4 PGUs), *m/z* 745 (6 PGUs) and *m/z* 869 (7 PGUs). They found that one specific molecular mass, corresponding to phlorotannin with 12 PGUs, had up to 61 isomers. Some isomers are shown in Fig. 5.4. This shows the complex nature of phlorotannins and emphasises the challenges involved in structural elucidation of these compounds.

Steevensz et al. (2012) used ultrahigh-pressure LC operating in hydrophilic interaction LC mode combined with high resolution MS for identification and characterisation of phlorotannins from five brown algae species. This approach proved to be accurate for profiling phlorotannins based on their degree of polymerisation. Similar sensitive methods with high mass accuracy and rapid full

mode scanning, e.g. methods with qTOF (time-of-flight) detector, are ideal tools for identification of phlorotannins.

The NMR techniques most useful for elucidating structures of phlorotannins include ^{1}H and ^{13}C NMR spectroscopy. With these methods the structures of over a hundred individual phlorotannins have been studied (Koch et al., 1980; Ragan, 1985; Nakamura et al, 1996). The combination of NMR and tandem MS is a strong tool in identification of phlorotannins. In theory, NMR can be used to verify MS data and the structural differences between phlorotannin isomers. However, this requires high degree of purification of the seaweed extracts to remove interfering co-extracted compounds. Hence, further development of analysis of individual phlorotannins would have to include isolation, purification and identification of compounds using preperative HPLC, MS and NMR.

5.4.   *In vitro* antioxidant properties of *F. vesiculosus* extracts

The antioxidant properties of phlorotannin rich *F. vesiculosus* extracts can be determined by antioxidant assays. Antioxidant assays are easy, inexpensive and fast methods to screen for antioxidant properties and have been used to map the antioxidant properties of the Nordic alga *F. vesiculosus* (Wang et al., 2009, 2012; Farvin & Jacobsen, 2013). The methods can be modified to fit microplates, which allows for multiple samples to be measured simultaneously.

Farvin & Jacobsen (2013) evaluated the antioxidant properties of e.g. ethanolic extracts from Danish *F. vesiculosus* using antioxidant assays. In the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and ferrous ion chelating assays they determined the effective concentration to inhibit 50% in the assay (EC_{50}) to be 9.9±1.6 and 1000.0±75.0 µg ethanolic extract /mL, respectively. For the reducing power they determined the absorption at 700nm at a concentration of 1 mg/mL (OD_{700}) to be 1.5±0.1. Wang et al. (2009) used DPPH radical scavenging and ferrous ion chelating assays to determine the activity of 70% acetonic and water extracts of Icelandic *F. vesiculosus.* Generally, 70% acetone extracted more compounds with scavenging abilities on DPPH radicals than water, with a 1/ EC_{50} of 90 (EC_{50} of $10.7*10^{-3}$ mg/mL) vs 60 for the water extract. In the ferrous ion chelating assay the water extract showed significantly higher activity than 70% acetone extract with an activity of nearly 100% vs. 55% for the 70% acetone extract at a concentration of 5 mg/mL. Wang et al. (2012) also used the above-mentioned assays to evaluate the antioxidant properties of phlorotannin rich fractions from Icelandic *F. vesiculosus*, e.g. the ethyl acetate fraction.

5.5.   Co-extracted antioxidative compounds

Other compounds than phlorotannins can influence the antioxidant properties of *F. vesiculosus* extracts. Farvin & Jacobsen (2013) found a correlation between high total phenolic content (TPC) and high radical scavenging of ethanolic *F. vesiculosus* extracts. However, no such correlation was found for water extracts although the radical scavenging activity was high for these extracts as well. Wang et al. (2009) studied the antioxidant properties of extracts from Icelandic *F. vesiculosus*. They found that the water extract had higher ferrous ion chelating activity than a 70% acetone extract and no correlation was found with the TPC in this assay. This suggests that other compounds with metal chelating ability such as polysaccharides, pigments, proteins or peptides are co-extracted with water. Hence, it appears that solvents used for extraction drastically influence the chemical composition of the extracts and their antioxidant properties.

Compounds like sulphated polysaccharides (fucoidans) are very polar compounds and can be extracted with water (Garrote et al., 1999). Fucoidans have shown radical scavenging activity, reducing power and metal chelating ability (Toth & Pavia, 2000; Rupérez et al., 2002). Proteins and peptides can also be co-extracted with water. Protein complexes from seaweed have been reported to possess metal chelating ability (Toyosaki & Iwabuchi, 2009). Furthermore, tocopherols have been found to be co-extracted in *F. vesiculosus* ethanolic extracts. Fucoxanthin is also more easily extracted with polar solvents than water due to its more lipophilic nature. Both tocopherols and fucxanthin can contribute to the radical scavenging activity in the extracts (Farvin & Jacobsen 2013). Moreover, vitamin E has shown synergistic activity together with seaweed extracts from *F. vesiculosus* and was related to the presence of chlorophyll *a* in the extracts (Le Tutour et al., 1998).

5.6.   Phlorotannin structure-antioxidant activity relationship

The antioxidant activity of phlorotannin compounds is, similar to phenolic compounds, assigned to the number and location of hydroxyl groups (Hatano et al, 1989). The oligomerisation of phloroglucinols seems to be crucial for the antioxidant activity. A few studies have investigated the antioxidant properties such as radical scavenging capacity of phlorotannins in brown seaweed extracts/fractions in order to relate this to their oligomerisation (Nakamura et al., 1996; Shibata et al., 2008; Audibert et al., 2010; Wang et al., 2012; Lee et al., 2012; Ferreres et al., 2012).

Nakamura et al. (1996) observed that fractions of high molecular weight phlorotannin oligomers exhibited higher antioxidant activity than fractions containing phloroglucinol and eckol (3 PGUs). Ferreres et al. (2012) also found stronger antioxidant activity of high molecular weight phlorotannins compared to low molecular weight phlorotannins, when studying six identified

phlorotannins from four different brown algae. Both Shibata et al. (2008) and Audibert et al. (2010) found contradicting results to this, as they observed that the radical scavenging activity of purified extracts of phlorotannins obtained from brown algae was related to the content of phlorotannins and to their molecular weight, and that the increase of molecular weight of the isolated phlorotannins leads to a decrease in antioxidant activity. Shibata et al. (2008) and Audibert et al. (2010) found that the radical scavenging activity of purified extracts of phlorotannins obtained from brown algae was related to the content of phlorotannins and to their molecular weight and that the increase of molecular weight of the isolated phlorotannins leads to a decrease of the antioxidant capacity..

Wang et al. (2012) fractionated phlorotannins from *F. vesiculosus* extracts based on molecular weight (size exclusion chromatography), and found no clear relationship between the antioxidant capacity (DPPH radical scavenging) and the degree of polymerization/molecular weight of phlorotannins. However, the HPLC-ESI-MS (electro spray ionization-mass spectrometry) analysis showed that the predominant phlorotannin of the most active fraction was an isomeric tetramer (fucodiphloroethol, *m/z* 499, $[M+H]^+$). Also, Lee et al. (2012) found no clear indication of a relationship between the size of the phlorotannins and the antioxidant activity against DPPH radicals. They isolated four antioxidative phlorotannins from *Ecklonia stolonifera*: 2-phloroeckol, eckol, phlorofucofuroeckol B, and 6,6'-bieckol by preparative HPLC, and evaluated the *in vitro* radical scavenging activity of the fractions obtained. They found significantly lower radical scavenging activity (EC_{50} of 35.2±0.4 μM) for 2-phloroeckol (4 PGUs), compared to the other phlorotannins (3 to 6 times higher EC_{50}) e.g. eckol and 6,6'-bieckol (6 PGUs).

5.6.1. On-line determination of antioxidant activity and characterization

Recent studies have suggested a method for determining antioxidant activity of specific compounds using a cyclic voltammetry approach (Arteaga et al., 2012; Pekal et al., 2012; Gulaboski et al., 2013; Lugonja et al., 2013). Cyclic voltammetry measures the ability of a compound to donate electrons and thus provides information of their antioxidant capacity. Plaza et al. (2014) found no significant difference in antioxidant capacity measured by cyclic voltammetry and radical scavenging assays when examining extracts from apple by-products. Zettersten et al. (2009) developed an on-line cyclic voltammetry method for characterisation of polyphenolic antioxidants in complex samples. This method was based on the combination of high-performance liquid chromatography with diode array, electrochemical, and tandem MS (HPLC-DAD-ECD-MS/MS).

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Chapter 6: Experimental work

This chapter gives an overview of the experimental design and approach used to fulfil the scientific aim of the PhD project. The work was divided into two main parts (Fig. 6.1), "Extraction and characterisation" (**Part I**) and "Application" (**Part II**), according to the hypothesis outlined in Chapter 1.
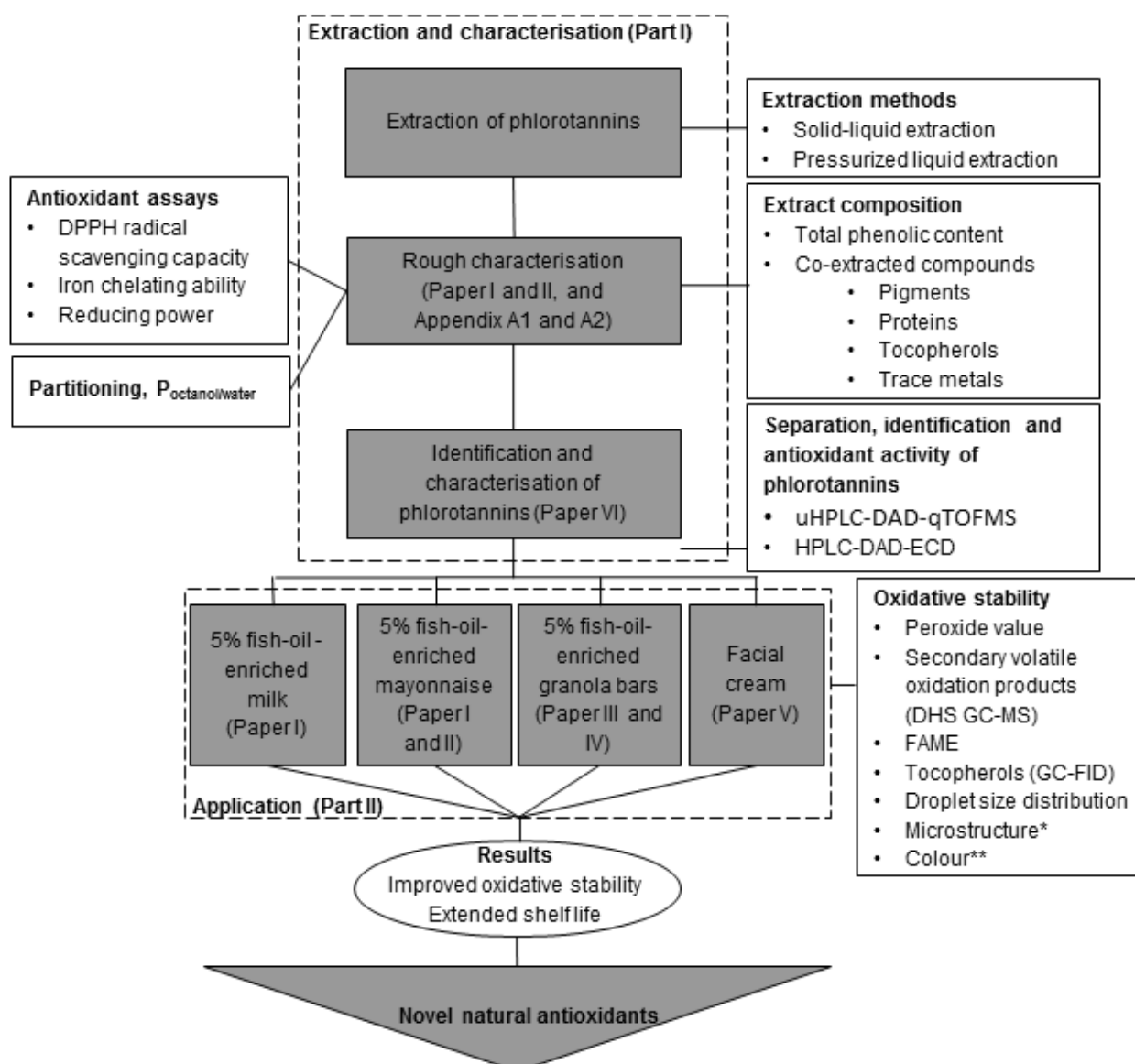
Fig. 6.1. Overview of the experimental design and methodology. *Microstructure (granola bars), **Colour (facial cream)

6.1. Part I: Extraction and characterisation

Extraction methods generating high amounts of phlorotannins were applied. The extracts were characterised in order to estimate the phlorotannin content as well as co-extracted antioxidant or prooxidant substances. Further identification of phlorotannins was performed using chromatographic techniques, including an on-line method to determine the antioxidant activity of individual phlorotannins. The methodologies are described in the following.

6.1.1. Extraction of phlorotannins

To ensure a high yield of phlorotannins, the collection of Icelandic *F. vesiculosus* was performed in early autumn (September 2011) in the Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland. Traditional solvent extraction (solid-liquid extraction (SLE)) was performed on this seaweed by Matís, the corporation partner in the project, and delivered to DTU Food for further analysis. The SLE method and solvent partitioning applied were according to Wang et al. (2009 and 2012). A study of the composition and antioxidant activity differences between the old (non-growing) and the young (growing tip) part of *F. vesiculosus* was also included, as extraction of WE was performed on a whole individual, and WoE and WyE was obtained from splitting the individual up into an old and young part, respectively.

Table 6.1. Extraction overview and extract abbreviations

Extraction method	Extract media	Code
Solid-liquid extraction	Distilled water	WE*
		WoE**
		WyE***
	Ethanol/water (80:20, v/v)	EE
	Acetone/water (70:30, v/v)	AE
Solvent partitioning	Ethyl acetate	EAF
Pressurized liquid extraction	Water	PLEW
	Ethanol/water (70:30, v/v)	PLEE

Solid-liquid extraction with water was performed on the *whole seaweed, on the **old part or on the ***young/growing part of the thallus

The Danish *F. vesiculosus* was likewise collected in early autumn (September 2009) where the phlorotannin content was at its maximum from a beach near Hou havn (55°54'39''N 10°14'59''E). This material was subjected to pressurised liquid extraction (PLE) to study the ability to extract phlorotannins in an alternative and more environmental friendly way compared to SLE. The study of PLE extracts was limited to a comparison of in vitro antioxidant activity and total phenolic content (TPC) with the SLE extracts and fraction, due to limited access to the extraction equipment

to obtain high amounts of extracts to be used in food and skin care model systems. The extracts obtained for the purpose are summarised in Table 6.1.

Solid-liquid extraction

Ethanol/water (80:20, v/v) and acetone/water (70:30, v/v) were used to extract phlorotannins from Icelandic *F. vesiculosus* according to Wang et al. (2009) with modifications. For more details on the SLE methods consult Paper I to VI.

Solvent partitioning

The crude extract 80% ethanol (EE) was fractionated by liquid-liquid partitioning (LLP) based on polarity as described by Wang et al. (2012). This resulted in an ethyl acetate-fraction (EAF) with increased phlorotannin content. More details on the fractionation is enclosed in Paper I.

Pressurized liquid extraction

In more "green" extraction approaches such as PLE, water can be used to extract phenolic compounds. Also, in PLE co-solvents such as ethanol can be used to increase the extraction of phlorotannins. Therefore, besides water, ethanol/water (70:30, v/v) was used to extract phlorotannins from Danish *F. vesiculosus.* The extraction method was based on Plaza et al. (2013) with modifications. The PLE method description is enclosed in appendix A.2.

6.1.2. Rough characterisation of extracts

Quantification of phlorotannins

Folin-Ciocalteu assay was applied to determine TPC of the extracts. Gallic acid was used to determine the gallic acid equivalent (GAE). For more details consult Paper I and II.

Partitioning of phenolic compounds

The partitioning of antioxidants in the different phases of emulsions is important for their antioxidant efficacy. Since it is hypothesised that the phlorotannins are the major antioxidants in *F. vesiculosus* extracts, the partitioning of phenolic compounds were determined in an octanol/water system, to simulate the partitioning in o/w emulsions. The phases were separated and the TPC of the water (TPC_w) and octanol (TPC_o) phase determined. TPC_i was the unknown and was estimated. The TPC of the different phases was converted to a percentage of the TPC of the extracts (TPC_{ex}).

$$TPC_i = TPC_{ex} - (TPC_w + TPC_o)$$

It should be taken into account that this study has a simplified approach, and did not consider the impact of factors like emulsifiers and their ability to influence the partitioning of antioxidants at the interface. For more details on the partitioning study refer to Paper II.

Determination of co-extracted compounds

Pigments, tocopherols, metal and protein content were determined in order to characterise the co-extracted compounds, which could possibly contribute to the overall antioxidant activity of the extracts. Only the SLE extracts and the EAF was submitted to co-extracted substance analysis. This was done due to the fact that these extracts were applied in food system and therefore a more thorough investigation of different antioxidant substances present in the extracts was needed. Detailed method descriptions are in Paper I and II.

Evaluation of antioxidant properties in vitro

DPPH radical scavenging, iron chelating and reducing power assays were used to determine the antioxidant properties of the extracts used in this PhD work. The antioxidant properties were determined at different concentration (Paper I and II and appendix A.2). Subsequently, the effective concentration to inhibit 50%, 30% or obtain an absorbance of 0.5 (EC_{50}, EC_{30} or $EC_{0.5}$) of the SLE extracts and the fraction was determined (appendix A.1). For details on the assays performed refer to Paper I and II and appendix A.1 and A.2.

DPPH radical scavenging assay

The efficacy of antioxidants in the DPPH radical scavenging assay is thought to be due to their H-donating ability. DPPH radical (DPPH•) is a stable free radical with a maximum absorbance at 517nm. It can accept an electron or H to become a stable diamagnetic molecule, DPPH-H. Butylated hydroxytoluene (BHT) is a well-known radical scavenger and is typically used to compare antioxidant efficacy when screening for radical scavenging activity by this assay.

Reducing power assay

The reducing power assay reflects the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in a redox linked colorimetric reaction, which can be measured at 700 nm. This property determines the tendency of the antioxidant to acquire electrons and thereby be reduced. Ascorbic acid is an example of a reducing antioxidant.

Iron chelating assay

Ferrozine is used in the ferrous ion (Fe^{2+}) chelating ability assay to form a stable complex with ferrous iron. This coloured complex can then be chelated by ferrous ion chelating antioxidants, resulting in reduced colour intensity with increased antioxidant activity. This can be measured at 562nm. EDTA is an example of an efficient iron chelating antioxidant.

6.1.3. Identification and characterization of phlorotannins

An on-line method to determine the antioxidant activity of specific phlorotannins was developed. The method concept was based on two approaches, a chromatographic separation and a cyclic voltammetry approach. The separation method was developed from the method applied by Safafar et al. (2015) for separation of phenolic compounds in microalgae. The cyclic voltammetry approach was based on the on-line cyclic voltammetry approach Zettersten et al. (2009) with some modifications. The method was applied on both HPLC-DAD-ECD and uHPLC-DAD-qTOFMS and chromatograms from the two analyses were compared to achieve tentative identification of phlorotannins and to determine their antioxidant activity. For more details consult Paper VI.

6.2.   Part II: Applications

Simple antioxidant assays are great screening tools in the search for potential antioxidants with relevant antioxidant properties. However, the reactions in the assays occur under simplified conditions, which do not consider the complexity of e.g. food and skin care products. Hence, the antioxidant effectiveness of potential antioxidants should not be evaluated only from the assays but needs to be verified in real food and skin care products as well.

The antioxidant evaluation of the SLE extracts and the LLP fraction was performed by conducting storage trials. The storage parameters are stated in Table 6.2. Concentrations of peroxide values and volatile secondary oxidation products were measured during storage to determine the oxidation level in the samples. In the following the approach and methodology of the storage trials are outlined. For more details on the production of the food and facial cream and the storage trials refer to Paper I to V.

6.2.1. Approach of evaluation of antioxidant efficacy in foods and skin care products

Milk, mayonnaise, granola bars and facial cream were used to evaluate the application potential of the SLE extracts and the LLP fraction from the Icelandic *F. vesiculosus* to meet the prerequisites of the project as described in section1.3. The application studies were conducted according to Table 6.2.

The concentrations of the extracts applied were based on results from preliminary *in vitro* antioxidant studies of the extracts. The concentrations used are stated in Table 6.2.

The foods used in the application studies were enriched with fish oil (FO) to enhance lipid oxidation and decrease the storage time. The first storage experiment included addition of WE and EAF to FO enriched milk. The milk system was the simplest system of the four. However, it was difficult to determine the antioxidant efficacy of the extracts in this type of food (will be elaborated in Chapter 7). Therefore, mayonnaise was used in the next study and all extracts were tested in this food due to initial promising results. Furthermore, antioxidant evaluation of selected extracts was performed with granola bars added both single and multi-layered FO emulsion systems.

Table 6.2. Application studies overview.

Type of product	Storage parameters	Extract added	Extract concentrations	Paper
0.5% FO enriched milk	12 days, 5°C, dark	WE	1, 1.5, 2 g/kg milk	I
		EAF	1, 1.5, 2 g/kg milk	I
15 % FO enriched mayonnaise	28 days, 20°C, dark	WE	1, 1.5, 2 g/kg mayo	I
		EAF	1, 1.5, (2) g/kg mayo	I
		WoE	1, 2 g/kg mayo	II
		WyE	1, 2 g/kg mayo	II
		EE	1, 2 g/kg mayo	II
		AE	1, 2 g/kg mayo	II
5% FO enriched granola bars (Trial I)	10 weeks, 20°C, dark	WE	0.5, 1 g/100 g emulsion*	III
		EE	0.5, 1 g/100 g emulsion*	III
		AE	0.5, 1 g/100 g emulsion*	III
5% FO enriched granola bars (Trial II)	10 weeks, 20°C, dark	WE	0.2 g/100 g emulsion**	IV
		EE	0.2 g/100 g emulsion**	IV
		AE	0.2 g/100 g emulsion**	IV
Facial cream	8 weeks, light, 40°C, dark/20°C	WE	1, 2 g/kg cream	III
		AE	1, 2 g/kg cream	III

*Concentrations added 70% FO/w emulsions which were subsequently added to the granola bars to obtain 5% FO enrichment. Total extract concentration in the bars was 0.4 g dw/kg and 0.7 g dw/kg, respectively
**Concentration added 25% FO/w emulsions which were subsequently added to the granola bars to obtain 5% FO enrichment. Total extract concentration in the bars was 0.4 g dw/kg

Possible applications of selected extracts were also studied in facial cream. To mimic the uncontrolled storage conditions that these types of products can be exposed to, the facial creams were stored under accelerated such as light exposure and elevated temperatures. Furthermore, colour changes during storage of commercially available creams containing Icelandic *F.*

vesiculosus extracts similar to the ones used in this PhD work have been observed but not further studied (UNA, 2016; Jónsdóttir , 2015). This colour change occurs even at room temperatures and could possibly be related to lipid oxidation. Therefore, it was of interest to determine the oxidative stability and colour changes to investigate if there were any correlation between the two when cream was stored dark and under ambient temperatures. EE and AE had showed similar properties and composition, thus we only found it necessary to evaluate one of these extracts (AE) in the facial cream model. Hence, as a counterpart to AE and WE were chosen.

6.2.2. Methodologies

The oxidative stability of the foods and the facial cream was determined by measuring different parameters related to lipid oxidation before and during storage. The methods used to determine oxidative stability are presented below. For a detailed description of the methods consult Paper I to V.

Primary oxidation products: Peroxide value

Primary oxidation products were determined by measuring lipid hydroperoxides. Peroxide values (PV) were determined according to the method by Shantha and Decker (1994), based on the formation of an iron-thiocyanate complex. The coloured complex was measured spectrophotometrically at 500 nm on either extracted lipids or the separated fat phase from the samples. The results are expressed in milli-equivalents peroxides per kg oil (meq O_2/kg).

Secondary oxidation products: DHS GC-MS

When lipid hydroperoxides are decomposed during the propagation step of lipid oxidation, volatile secondary oxidation products are formed. The secondary volatile oxidation compounds were collected by dynamic headspace (DHS) by a purge trap method on GR™ packed tubes as described by Hartvigsen et al. (2000), Let et al. (2003) and Horn et al. (2009). The volatile compounds were analysed using gas chromatography (GC) MS. The quantification of the volatile compounds relied on calibration curves prepared from external standards. For milk and mayonnaise the calibration curves were performed in identical systems though without adding neither FO nor seaweed extracts, thus taking the release of volatile compounds from the matrix into account. For granola bars and facial cream the calibration curves were based on direct trapping of standard volatiles in the Tenax tubes. Results were given in ng/g of food or facial cream.

Fatty acid composition and tocopherol content

During the storage period other product parameters can change as a result of lipid oxidation, e.g. fatty acid composition and tocopherol content.

The fatty acid composition was determined by the FAME method and analysed by GC-FID according to AOCS (1998). Results were given in % and a *Relative Decrease in Area%* (RDA%).

Tocopherols are determined by HPLC to identify the tocopherol homologues present and their quantities (AOCS, 1998). The results were given as µg tocopherol/g dw.

Droplet size distribution

The size of oil droplets in emulsion systems can influence the lipid oxidation rate. Hence, the size of fat globules was determined in all emulsions (milk, mayonnaise, 70% FO/w emulsion and facial cream) by laser diffraction. Milk was measured directly while other emulsions were diluted before being analysed. In milk, mayonnaise and facial cream the droplet size distribution (DSD) was determined at the beginning and end of storage to investigate how this changed during storage. DSD was only measured at day 0 for the 70% FO/w emulsion as this was then added to granola bars. The results were given as surface area mean diameter $D[3,2] = \sum d^3/d^2$ according to Rawle (1996).

Microscopy of granola bars

Knowledge regarding the incorporation of the FO/w preemulsions in granola bars and their physical microstructure, which may be affected by the addition of antioxidant, is needed to understand how the oxidative stability depends on the physical location of the emulsions in the granola bars. Therefore, the physical location of the 70% FO/w preemulsions added *F. vesiculosus* extracts in the granola bars was determined by three microscopic methods: traditional scanning electron microscopy (SEM), environmental scanning electron microscopy (ESEM) and/or confocal light scanning microscopy (CLSM). For more details on the microscopic methods applied refer to Paper III and IV.

Colour changes in facial cream

The colour changes were determined in all facial creams with or without the extracts. Colour was measured using a digital colorimeter to obtain the colour coordinates L*, a* and b*. These values were used to calculate the euclidean distance value of facial cream during storage:

$$\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}$$

6.3. Data treatment and statistics

Univariate statistical methods were employed in all studies. These methods included one-way and/or two-way ANOVA, followed by a multiple comparison test to determine significant difference between individual samples and/or sampling points. In some cases data was subjected to

multivariate data analysis, e.g. principal component analysis (PCA). Multivariate tools were applied to give an overview of the variance in the samples.

Furthermore, the inhibition% of the extracts from day 0 to the end of storage was calculated for PV and one representative secondary volatile oxidation product (1-penten-3-ol (FO enriched food models) or heptanal (facial cream model)) for all extract applications. In the calculation a higher PV or volatile amount at day 0 is accounted for. The calculation was as follow:

$$\%Inhibition = 100 - \left(\left(\frac{S_x - S_0}{C_x - C_0} \right) * 100 \right)$$

S_x is the measured value of a sample at day X. S_0 is the measured value of a sample at day 0. C_x is the measured value of the control at day X, and C_0 is the measured value of the control at day 0. The %inhibition of the extracts is stated in Table 7.5 in Chapter 7. The antioxidant has a prooxidative effect if the inhibition < −5% and an antioxidative effect >5%. The antioxidative effect can be categorized as weak (5–20%), intermediate (20–50%) or strong (>50%) effects (Jacobsen et al., 2008).

6.4.   References

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Chapter 7: Results and discussion

This chapter summarises the main findings and discusses the results in relation to relevant literature and the scientific hypothesis proposed in Chapter 1. For more detailed information about the results consult the papers and appendices.

The structure of this chapter is as follows:

1. Part I: Extraction and characterisation
   - Rough characterisation of SLE extracts and LLP fraction (Paper I and II, and Appendix A1)
     - Quantification of phlorotannins
     - Partitioning of phenolic compounds
     - Co-extracted antioxidative and/or proxidative compounds
     - Antioxidant properties
   - Identification and Characterisation of phlorotannins (Paper VI)
     - Structural elucidation of phlorotannins
     - On-line antioxidant activity of individual phlorotannins
   - Evaluation of PLE for extraction of phlorotannins (Appendix A2)

2. Part II: Application
   - Antioxidant efficacy of Icelandic *F. vesiculosus* extracts in fish-oil-enriched foods
     - In milk (Paper I)
     - In mayonnaise (Paper I and II)
     - In granola bars
       - Trial 1 (Paper III)
       - Trial 2 (Paper IV)
   - Antioxidant efficacy of Icelandic *F. vesiculosus* extracts in skin care products
     - In facial cream model (Paper V)

7.1.   Part I: Extraction and characterisation

7.1.1. Rough characterisation of SLE extracts and LLP fraction from F. vesiculosus
The aim of the extraction was to obtain high amounts of phlorotannins from the Icelandic *F. vesiculosus* and hereafter determine the antioxidant activity of the extracts to the phlorotannins. It has been suggested that the antioxidant activity of *F. vesiculosus* extracts is not only due to the

presence of phlorotannins, but also to antioxidative co-extracted compounds (see section 4.4). Therefore, it was crucial to determine the composition of the extracts to get more knowledge about which other compounds might influence the antioxidant activity.

Quantification of phlorotannins

Firstly the phlorotannin content was estimated by the Folin–Ciocalteu assay. The results are shown in Table 7.1.

Table 7.1. Extract characterisation of F. vesiculosus SLE extracts and LLP fraction. Total phenolic content (TPC) [g GAE/100g dw] and in vitro antioxidant properties determined by $1/EC_{XX}$ values (DPPH radical scavenging, Iron chelating ability and Reducing power). Mean ± SD

SLE extract	LLP fraction	TPC [g GAE/100g dw]	Antioxidant properties		
			DPPH radical scavenging capacity [mg dw/mL]	Iron chelating ability [mg dw/mL]	Reducing power [mg dw/mL]
			$1/EC_{50}$ ± SD	$1/EC_{30}$ ± SD	$1/EC_{0.5}$ ± SD
Water, WE*		18.4±0.1[c]	53.0±3.6[b]	2.5±0.2[c]	5.1±0.3[c]
Water (old), WoE**		6.9±0.3[a]	13.8±1.0[a]	0.6±0.1[a]	2.2±0.0[a]
Water (young), WyE***		12.6±2.1[b]	16.9±2.2[a]	0.5±0.1[a]	4.0±0.1[b]
Acetone, AE		23.2±1.1[d,e]	68.2±0.3[b]	1.5±0.1[b]	8.4±0.2[d]
Ethanol, EE		20.4±2.4[c,d]	75.3±15.4[b]	2.1±0.3[c]	7.9±0.0[d]
	Ethyl acetate, EAF	26.5±1.2[e]	169.3±34.8[c]	0.3±0.0[a]	13.5±0.4[e]

*Whole seaweed, **old part and ***young/growing part of the thallus
Different letters in the same row indicates significant differences between samples in a specific assay

It was possible to extract phenolic compounds from *F. vesiculosus* by solid-liquid extraction (SLE). Among the SLE extracts TPCs ranged from 6.9 ± 0.3 g GAE/100 g dw in WoE to 23.2 ± 1.1 g GAE/100 g dw in EE. TPC was different between WE, AE and EE, indicating a variation in extraction efficiency depending on the solvents in the order: water≤ethanol<acetone. Moreover TPC varied significantly ($p < 0.05$) between water extracts and was dependent on which part of the seaweed the extraction was performed on, where the oldest part (WoE) had the lowest TPC. The LLP fraction, EAF, obtained from EE had the highest TPC of 26.5 ± 1.2 g GAE/100 g dw, indicating successful purification of phlorotannins. The sum of TPC for WoE and WyE was approximately equal to the TPC of WE, indicating that the TPC of old thallus and the young leaves together equals the TPC of the whole seaweed.

When comparing the TPC values of extracts obtained by Wang et al. (2009) using the same solvents for extraction of phenolic compounds from Icelandic *F. vesiculosus*, it was found that Wang et al. (2009) in general obtained higher TPC values. Also, Wang et al (2012) obtained higher TPC of an ethyl acetate fraction (88.3 ± 2.2 of PGEs/100 g) similar to EAF also from Icelandic *F.*

vesiculosus. The higher TPC found by Wang et al (2009 and 2012) contra what we found, can be due to seasonal variation in the TPC of *F. vesiculosus*, as they collected *F. vesiculosus* in March and October, respectively.

We found that the phenolic content of the young part of the seaweed (WyE) was higher than the old part (WoE). Whether young leaves of *F. vesiculosus* generally have higher TPC compared to old leaves, is interesting regarding a sustainable production of *F. vesiculosus* since harvesting of only the young part of the seaweed and not a full removal of the seaweed, will result in a continued reproductive growth of the parent plant. Hence, this has to be further investigated.

Partitioning of phenolic compounds

The efficacy of antioxidants is highly influenced by their location in o/w emulsions. Preferably, the antioxidants should be localised at the interface between water and oil. This requires amphiphilic properties of the antioxidants or interactions of the antioxidants with surface active compounds, e.g. proteins. The octanol/water partitioning coefficient ($P_{o/w}$) for the TPC was determined for the SLE extracts and LLP fraction (further referred to as extracts). The approach was to determine the phenolic compound in the octanol and water phase, and at the interface by calculations based on TPC of the extracts (see approach in Chapter 6). The distribution of phenolic compounds in the water, octanol or interface is shown in Fig. 7.1.

The highest amount of phenolic compounds was found at the interface, indicating that the phenolic compounds have high affinity to the amphiphilic area between octanol and water. EAF had the highest amount of phenolic compounds located at interface (22.1 g GAE/100 g EAF) followed by, AE (19.1 g GAE/100 g AE), EE (16.5 g GAE/100 g EE), WE (14.9 g GAE/100 g WE), WyE (9.5 g GAE/100 g extract) and WoE (2.5 GAE/100 g extract). The high partitioning of phenolic compounds at the interface could be due to interactions between phenolic compounds and co-extracted surface active compounds, e.g. proteins, present in the extracts as described by Porter (1989). However, no proteins were detected in the EAF (Fig. 7.2) indicating a more water soluble nature of the proteins in *F. vesiculosus*. Hence, it is more likely that the interfacial partitioning of phenolic compounds in EAF is due to extraction of phenolic compounds with amphiphilic properties. It can be hypothesized that the presence of phenolic compounds in the interface could increase the antioxidant activity in o/w emulsions because the phlorotannins may cover the oil droplets and protect them against radicals and oxidation initiators from the water phase.
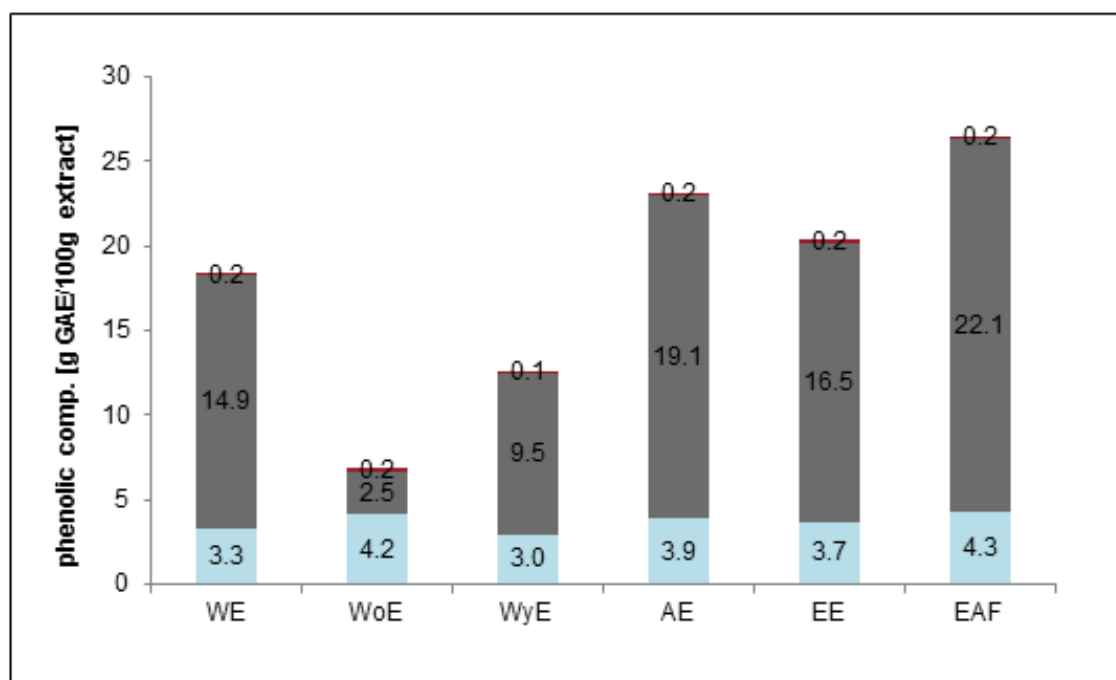
Fig. 7.1. Partitioning of phenolic compounds. Distribution of phenolic compounds (g GAE/100g) in the different SLE extracts (WE, WoE, WyE, AE and EE) and the LLP fraction (EAF) in octanol (red), water (blue) and interface (grey)

Determination of co-extracted antioxidative and/or proxidative compounds

Besides extraction of phenolic compounds, a wide range of compounds with potential antioxidant or prooxidant properties were co-extracted during the extraction process (Table 7.2).

The protein content was below detection limit in WE and EAF, but was found to be up to 7.2 ± 0.0 % (w/w) in WoE. The protein content in WoE and WyE was higher than that in EE and AE. The higher content of protein in the water extracts might be due to a high level of hydrophilic proteins in *F. vesiculosus*, and therefore, these proteins will co-extract with water to a larger extent than with ethanol or acetone, and will then be fully lost in the purification with ethyl acetate. High protein content was observed in WyE and WoE. However, no proteins were extracted in the WE, where it was expected that the protein content of WE would be equal to the sum of WoE and WyE as this was observed for TPC in Table 7.1. For the determination of co-extracted compound, a higher total sum of WoE and WyE compared to the total of WE, was found. This indicated that there was large diversity in the composition of co-extracted compounds between *F. vesiculosus* individuals even though harvesting was performed the same month the same year at the same location.

Table 7.2. Antioxidant and prooxidant constituents in F. vesiculosus SLE extracts and the LLP fraction. Protein content [%], tocopherol content [µg toc/g dw], pigments [µg/mg dw] and trace metal content (iron and cupper) [µg/g dw]

			WE	WoE	WyE	AE	EE	EAF
Protein		% (w/w)	nd*	7.2±0.0	4.6±0.2	2.3±0.0	1.7±0.0	nd*
Tocopherol		*Alpha*	19.0±1.9	42.2±4.4	11.5±4.9	4.0±0.3	2.4±1.9	8.3±0.1
		Beta	2.9±0.0	5.2±0.5	2.6±1.1	1.9±0.7	1.7±0.3	3.5±0.0
		Gamma [µg toc/g dw]	6.2±0.2	10.4±1.0	3.0±1.2	2.5±0.8	1.8±0.5	5.1±0.6
		Delta	24.5±1.2	40.2±4.1	13.0±6.0	12.9±0.6	18.0±4.4	43.4±2.0
Pigments	Chlorophylls	*Chlorophyll C2*	0.5±0.0	1.1±0.1	0.6±0.0	0.6±0.1	1.5±0.2	15.8±2.6
		Chlorophyllide a [µg/mg dw]	nd*	nd*	nd*	nd*	0.5±0.0	10.0±1.5
		DV Chlorophyll a	nd*	2.7±0.1	0.8±1.1	nd*	nd*	nd*
		Chlorophyll a	nd*	0.1±0.2	nd*	0.2±0.0	nd*	nd*
	Carotenoids	*19-But-Fucoxanthin*	nd*	2.9±0.1	1.3±0.1	0.4±0.1	4.9±0.1	43.0±1.9
		Fucoxanthin	nd*	nd*	0.6±0.8	nd*	0.9±0.4	9.4±3.7
		Prasinoxanthin	1.3±0.0	1.4±0.0	0.3±0.1	nd*	nd*	4.0±0.4
		19-Hex-fucoxanthin	nd*	0.3±0.1	0.2±0.0	0.2±0.0	0.7±0.1	nd*
		Astaxanthin	nd*	0.5±0.1	0.2±0.0	nd*	0.9±0.2	nd*
		Diadinoxanthin [µg/mg dw]	0.2±0.04	nd*	nd*	nd*	nd*	6.4±0.1
		Dinoxanthin	0.2±0.1	0.4±0.0	0.2±0.0	nd*	nd*	4.7±0.4
		Zeaxanthin	0.2±0.1	0.4±0.3	nd*	0.2±0.0	0.2±0.1	nd*
		Lutein	nd*	nd*	0.4±0.0	nd*	nd*	0.7±0.0
		Dihydrolutein	0.3±0.0	nd*	nd*	nd*	nd*	nd*
		Carotene a+b	1.7±0.7	2.4±0.0	0.6±0.8	nd*	nd*	1.0±0.3
Trace metals		*Fe* [µg/g dw]	4.4±1.0	61.5±0.4	35.2±0.5	9.5±1.1	14.5±0.1	85.5±1.2
		Cu	0.9±0.0	2.0±0.0	1.0±0.2	1.2±0.0	0.5±0.0	1.6±0.2

*nd, not detected

All four extracts contained tocopherol. This finding is in agreement with the findings of Farvin and Jacobsen (2013) who found that brown seaweed, especially species of *Fucus*, contained high amounts of alpha, beta, gamma and delta tocopherols. The content of alpha tocopherol was higher in all water extracts compared with EE and AE. The high amounts of tocopherol observed could contribute to the radical scavenging properties of the extracts as described in section 4.4.

Iron and cupper (Fe and Cu) were determined to estimate the amount of possible prooxidants in the extracts. All extracts contained both metals, and iron was dominant ranging from 4.4±1.0 to

61.5±0.5 µg/g dw in the SLE extracts and was highest in the LLP fraction, EAF. Since EAF is a fraction of EE, the higher iron content in EAF compared to EE indicated that the metals are partitioning into the ethyl acetate during purification. The metal content of *F. vesiculosus* can varies depending on the concentration of these metals in the ambient water. The iron content of *F. vesiculosus* from the Øresund region (Kattegat to Baltic) between Denmark and Sweden has been found to vary from 53 to 151 µg iron/g dw depending on location (Phillips, 1979). These values from seaweed plants were thus higher than the iron content in the extracts, indicating that the ambient waters in Iceland might contain lower levels of metals compared to Øresund.

The pigments identified and quantified belonged to the main groups of carotenoids or chlorophylls similar to what was described in section 4.4. When estimating the total amount of chlorophylls and carotenoids in the SLE extracts, WoE and EE had the highest amount of both pigment groups. Fucoxanthin was the dominant carotenoid. EAF contained a greater variety of pigments and also larger amounts compared to the SLE extracts. The purification step of EE also seemed to concentrate the pigments, especially chlorophyll C2 and fucoxanthins (19-But-Fucoxanthin and fucoxanthin). Pigments, e.g. diadinoxanthin and dinoxanthin, which were below detection limit in EE, were found in EAF. The amounts of specific pigments in Nordic *F. vesiculosus* (Baltic and North Atlantic) was previously reported to be 5 µg chlorophyll a /mg dw and 1 µg fucoxanthin /mg dw (Nygard and Ekelund, 2006). This is in accordance with our results for the fucoxanthin in EE, but for the chlorophyll a, our results were much lower. It should though be considered that the extraction of pigments performed by Nygård and Ekelund (2006) aimed at extracting chlorophylls and carotenoids (with 90% acetone) where we aimed at extracting phlorotannins. Chlorophylls as well as oxidised or breakdown products hereof can have severe pro-oxidative effects, since they can work as sensitizers in photo oxidation and lead to formation of singlet oxygen (Andersen & Skibsted, 2010). The amount of carotenoids, such as fucoxanthin and lutein was particularly high in EAF. Fucoxanthin and lutein have been recognised as antioxidants in autoxidation due to their high radical scavenging activity (Le Tutour et al., 1998; Sindhu et al., 2010). Furthermore, Haila et al. (1996) found that a combination of lutein and γ-tocopherol was synergistic in inhibiting autoxidation of triacylglycerides.

Antioxidant properties

In Paper I and II the antioxidant properties of the SLE extracts and LLP fraction were investigated at different concentrations (1.0, 1.5 and 2.0 mg dw/mL water). In a follow up study, $1/EC_{50}$, $1/EC_{30}$ and $1/EC_{0.5}$ were determined for the DPPH radical scavenging capacity, iron chelating ability and the reducing power, respectively. The approach of the follow up study is presented in Appendix A.1 and the results are shown in Table 7.1.

According to the determined EC values for the antioxidant properties, the DPPH radical scavenging capacity of the extracts was dominant, whereas the extracts were less efficient iron chelators. EAF exhibited the highest activity in this assay. A positive correlation between TPC and radical scavenging capacity was observed. It was observed that TPC of EAF was approximately twice as high as for WyE, while the radical scavenging capacity was ten times higher. This indicates that the relationship between TPC and radical scavenging capacity was not proportional. Similar correlation between TPC and radical scavenging capacity has previously been observed (Wang et al. 2009 & 2012). The radical scavenging capacity and reducing power showed similar trends, as extracts with high radical scavenging capacity also showed high reducing power. This has previously been observed (Farvin and Jacobsen, 2013).

The ferrous ion chelating ability of the extracts showed very different patterns compared with both radical scavenging activity and reducing power and no correlation between TPC and iron chelating ability was observed. WE and EE were more efficient in chelating ferrous ions compared to the other extracts. Thus, purification of EE by LLP with ethyl acetate compromised for the iron chelating ability of EAF. Hence, it can be assumed that ethyl acetate removes compounds with iron chelating ability, e.g. polysaccharides.

Wang et al. (2009) and Farvin and Jacobsen (2013) also found higher iron chelating ability of water extracts despite a lower phenolic content compared with other extracts (ethanol and acetone extracts). Highly polar compounds, other than phenolic compounds, with iron chelating ability must therefore be co-extracted by water and contribute to the iron chelating ability. Toth and Pavia (2000) reported that compounds such as sulphated polysaccharides (fucoidans) and/or phytochelatins were more effective than phlorotannins for the detoxification to copper accumulation in the brown alga *Ascophyllum nodosum*, indicating that these compounds can chelate metals. These compounds are presumably not extracted with ethyl acetate, since a decrease in iron chelating ability of EAF compared to EE was observed.

Summary of correlation between composition and in vitro antioxidant properties
No previous studies have performed similar characterisation of the composition of *F. vesiculosus* extracts as well as related the composition of both phlorotannins and co-extracted antioxidative and prooxidative compounds to the *in vitro* antioxidant properties. A PCA was performed on the data to study the correlation between extract composition and *in vitro* antioxidant properties of the extracts (Fig. 7.2).
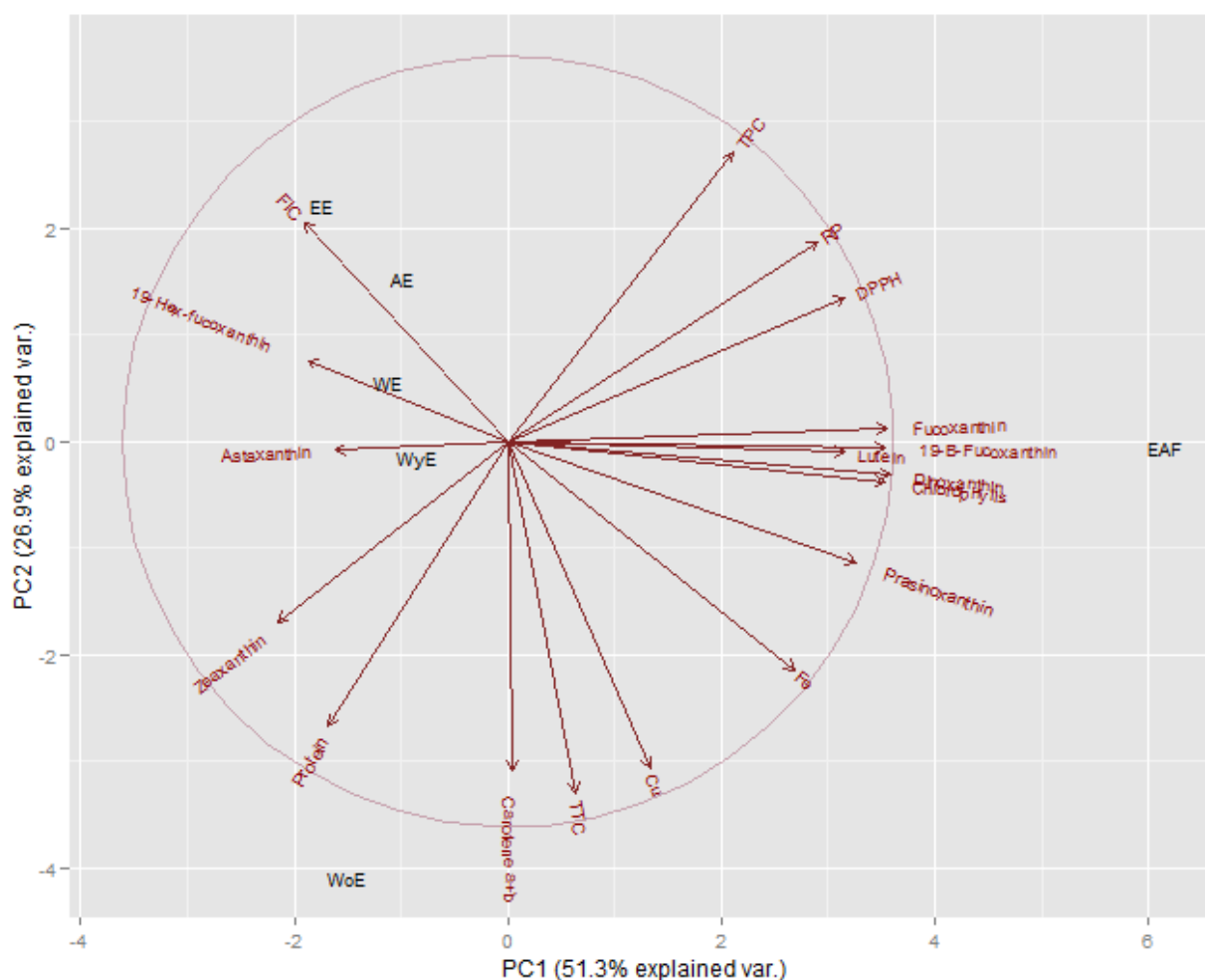
Fig. 7.2. Principal component analysis (PCA). Bi-plot incl. scores (extracts) and loadings variables (characterisation parameters) abbreviations: TPC=total phenolic content, TTC=total tocopherols content, FIC=ferrous ion chelating, RP=Reducing power, DPPH=DPPH radical scavenging capacity

The correlation between TPC and DPPH radical scavenging capacity was emphasised by the PCA model as these two variables, including reducing power, were closely related. Regarding pigments, the different pigments are spread all over the PCA loadings plot which made correlations to the antioxidant properties difficult. Nevertheless, it cannot be ruled out, that pigments like fucoxanthin (fucoxanthin and 19-B-fucoxanthin) and lutein influenced the DPPH radical scavenging and reducing power of the extracts, as these two pigments to a higher degree was correlated to these *in vitro* antioxidant properties compared to other pigments. EAF was correlated with these two pigments as well as chlorophylls, TPC, radical scavenging capacity and reducing power.

The pigment 19-Hex-fucoxanthin was correlated with the ferrous ion chelating ability. AE, EE and WE were all correlated with ferrous ion chelating ability and 19-Hex-fucoxanthin. Moreover, AE and

EE were also found to be correlated to TPC. WyE was correlated with astaxanthin and WoE was correlated more to proteins, tocopherols, carotene a+b and trace metals.

The results indicate that overall the phlorotannins are the main contributor to high antioxidant activity, especially high radical scavenging activity and high reducing power. Therefore, further identification and characterization of these highly antioxidative polyphenolic compounds are of interest.

7.1.2. Identification and characterisation of phlorotannins

Structural elucidation of phlorotannins

EAF was the purified fraction of EE and was found to have high TPC and high radical scavenging activity. It was assumed that EAF had high content of phlorotannins and therefore we continued working with this fraction for structural elucidation of phlorotannins. For more details on this study consult Paper VI.

The Extracted Ion Chromatograms (EIC) of deprotonated molecular ions ([M-H]$^-$) from the most common phlorotannins found in literature (eckol (*m/z* 371.0409), fucophloroethol (*m/z* 373.0565), 7-phloroeckol (*m/z* 495.0569), fucodiphloroethol (*m/z* 497.0725), phlorofucofuroeckol (*m/z* 601.0624), fucotriphloroethol (*m/z* 621.0886), dieckol (*m/z* 741.0733), and fucophloroethols with six (*m/z* 745.1046), and seven PGUs (*m/z* 869.1207)), was used for the study of phlorotannins in EAF by UHPLC-DAD-qTOFMS. Furthermore the elemental compositions were verified by the accurate mass (± 5 ppm). Furthermore, the MS fragmentations of any other peaks observed by UV were also studied.

In Fig. 7.3 the Base Peak Chromatogram (BPC) of EAF is shown together with the UV chromatogram and EICs. In the EICs, some peaks were overlapping due to insource fragmentation (i.f.) giving false/positive results when consulting MS-data, e.g. one peak in EIC of *m/z* 373 showed to be an insource fragmentation of *m/z* 497. When taking this into account the EICs revealed well-defined and abundant ions of 11 (1-11) compounds tentatively corresponding to phlorotannins and corresponding with the UV chromatogram, and 2 compounds (12-13) which were only found in trace amounts and were not so well-defined, neither by UV. There were no responses in the UV chromatogram, which did not correspond to the studied ions.
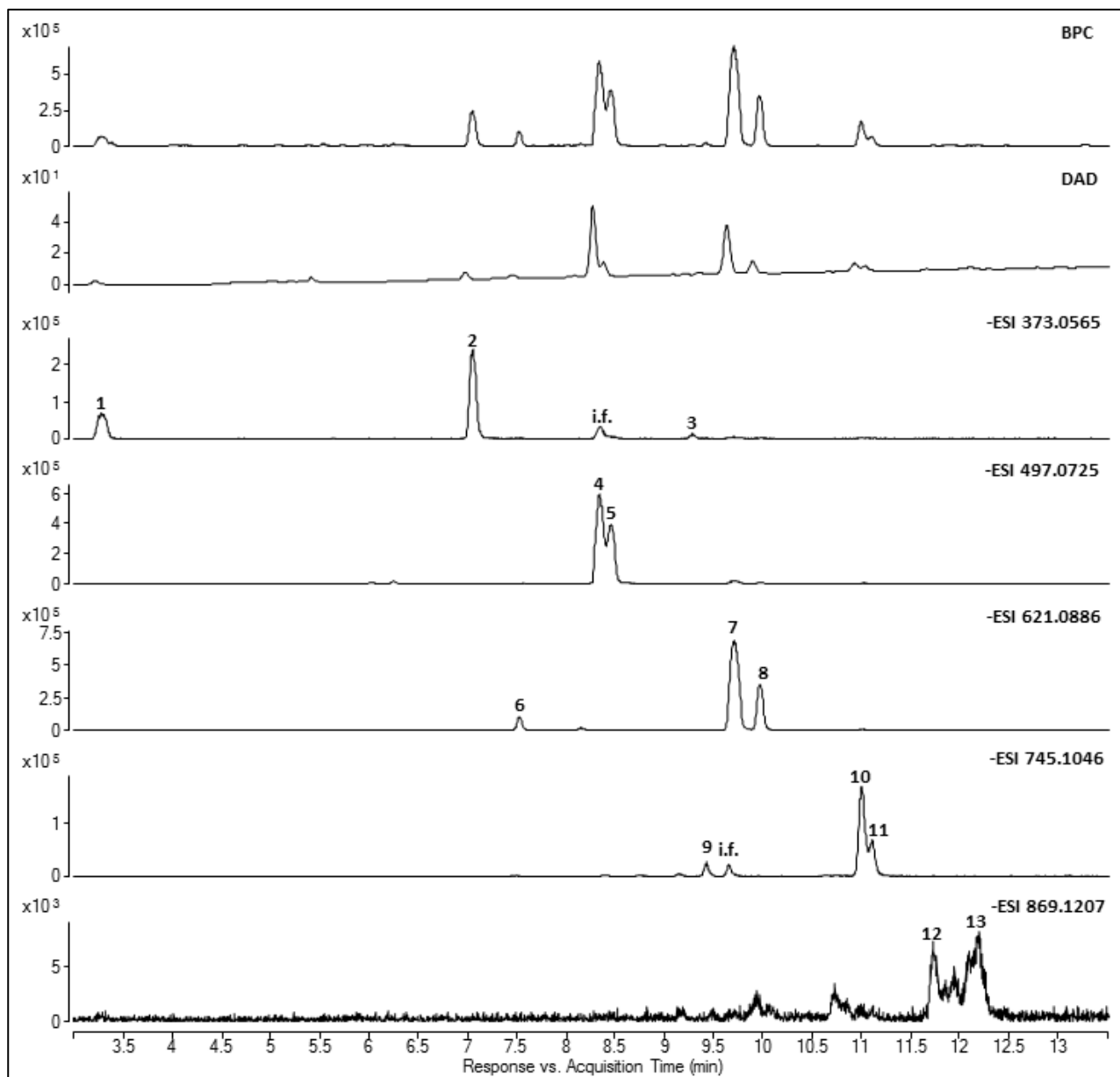
*Fig. 7.3. Base Peak chromatogram (BPC), UV chromatogram (DAD) and Extracted Ion Chromatograms (-EIC) of phlorotannins from EAF. [M-H]⁻ monoisotopic m/z: **1-3**,373.0565; **4-5**, 497.0725; **6-8**, 621.0886; **9-11**, 745.1046; **12-13**, 869.1207 (± 5 ppm). i.f.=insource fragmentation*

The MS study of the ions allowed the detection of several isomers. The isomers were studied in both negative and positive ionization mode (-EIS, +EIS), to investigate the fragmentation patterns to see if it was possible to come closer an exact structural identification of the isomers. Fig. 7.4 shows the fragmentation patterns of the two isomers, compound 7 and 8, with molecular ions in both negative ([M-H]⁻, m/z 621.0859) and positive mode ([M+H]⁺, m/z 623.1019). Fragmentation in negative ionisation mode gave well-defined fragmentation of the phlorotannins compounds. Hence, the further structural elucidation was studied in this mode.

Compounds 7 and 8, in negative mode, showed similar fragmentation patterns in which some ions are characteristic of phlorotannins fragmentation, e.g. with losses of one and two water molecules (-18 (m/z 603) and -36 (m/z 585), respectively), loss of 1 PGU and water (-126, -18 (m/z 477)), loss of 2 PGUs and water (-2PGUs, -18 (m/z 353)), as well as the presence of deprotonated molecular ion of phloroglucinol (m/z 125). Thus, compound 7 and 8 (and 6) is suggested to be phlorotannins composed of five PGUs, possibly isomers of fucotriphloroethol. In Fig. 7.5 suggested major fragmentation of fucotriphloroethol (linear) is shown. It is most likely the loss of one and two water occurs first, followed by fragmentation from the ether-end due to the higher lability of this bond compared to the phenyl-linkage.

The MS study of the ions allowed the detection of several isomers. The isomers were studied in both negative and positive ionization mode (-EIS, +EIS), to investigate the fragmentation patterns to see if it was possible to come closer to an exact structural identification of the isomers. Fig. 7.4 shows the fragmentation patterns of the two isomers, compound 7 and 8, with molecular ions in both negative ([M-H]$^-$, m/z 621.0859) and positive mode ([M+H]$^+$, m/z 623.1019). Fragmentation in negative ionisation mode gave well-defined fragmentation of the phlorotannin compounds. Hence, further structural elucidation was studied in this mode.

Compounds 7 and 8, in negative mode, showed similar fragmentation patterns in which some ions are characteristic of phlorotannin fragmentation, e.g. with losses of one and two molecules of water (-18 (m/z 603) and -36 (m/z 585), respectively), loss of 1 PGU and water (-126, -18 (m/z 477)), loss of 2 PGUs and water (-2PGUs, -18 (m/z 353)), as well as the presence of a deprotonated molecular ion of phloroglucinol (m/z 125). Thus, compound 7 and 8 (and 6) is suggested to be phlorotannins composed of five PGUs, possibly isomers of fucotriphloroethol. In Fig. 7.5 a suggested major fragmentation of fucotriphloroethol (linear) is shown. It is most likely that the loss of one and two water molecules occurs first, followed by fragmentation from the ether-end due to the higher lability of this bond compared to the phenyl-linkage.
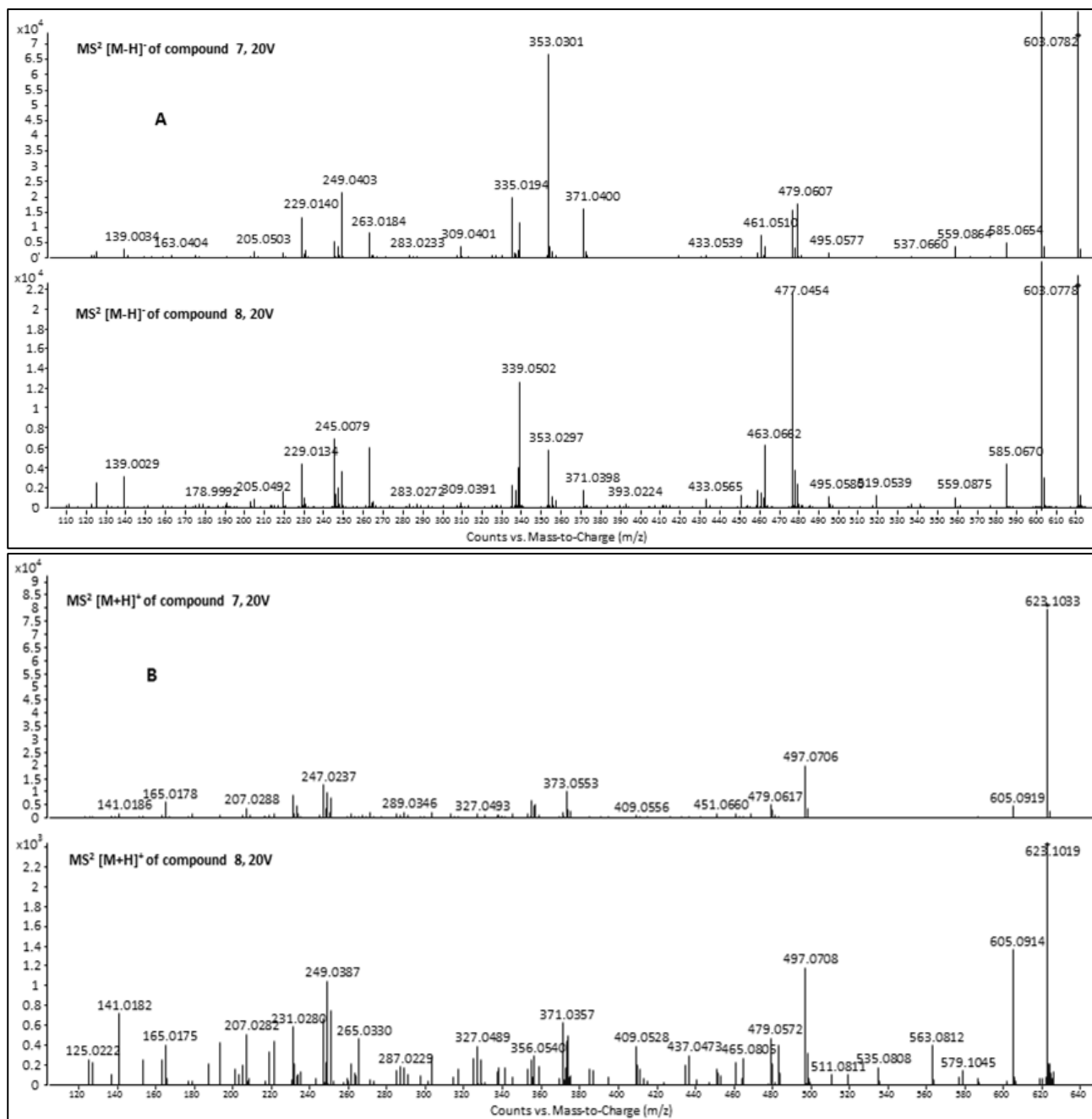
Fig. 7.4. MS/MS of compound 7 and 8 (5 PGUs). A: –EIS, [M-H]⁻, m/z 621.0859, and B: +EIS, [M+H]⁺, m/z 623.1019
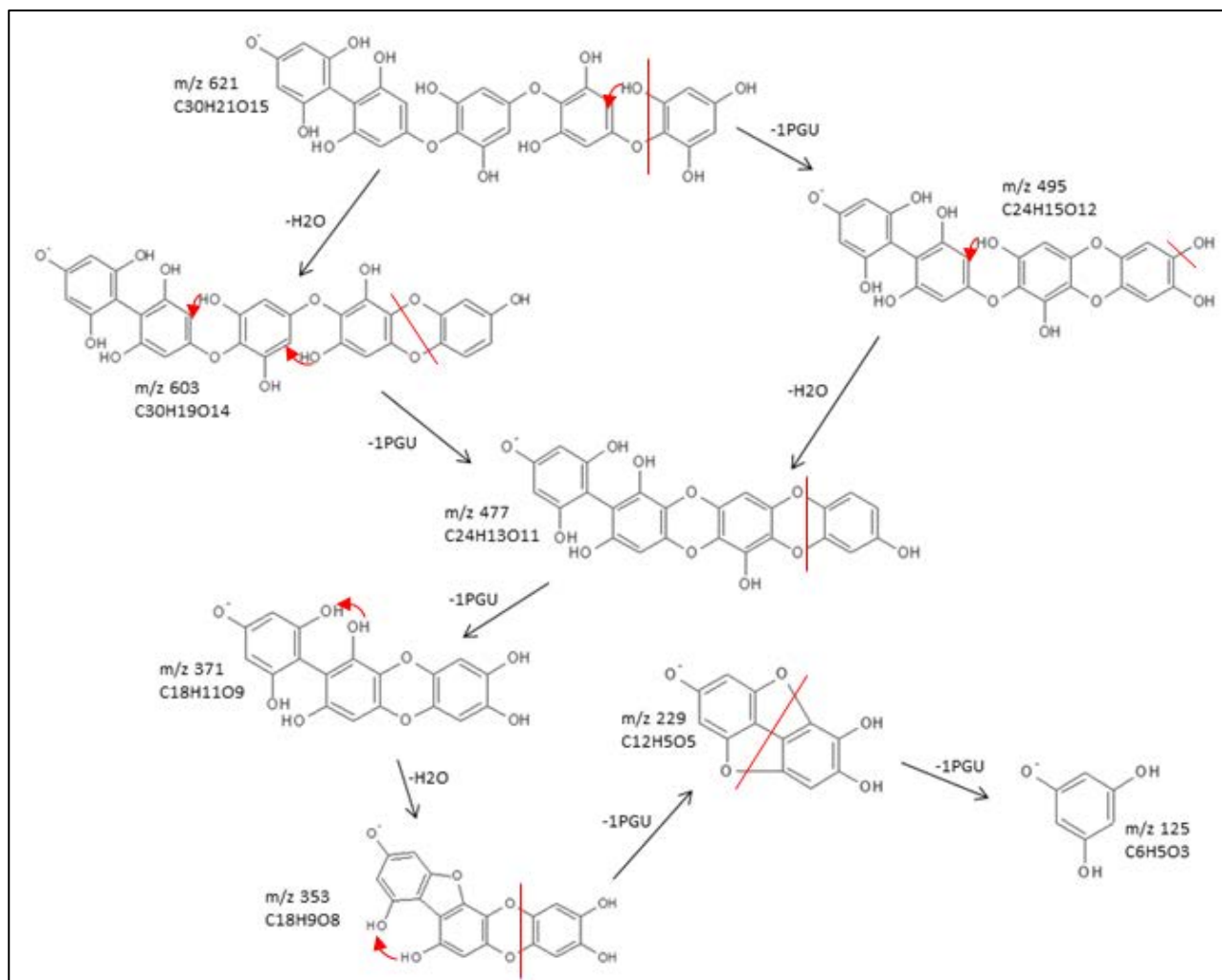
Fig. 7.5. Fragmentation suggestion of the hypothesized structure of compound 8 (Fucotriphloroethol, 5 PGUs) according to MS/MS data of [M-H]⁻, m/z 621.0859

In Table 7.3 the fragmentation patterns in negative mode of the 13 identified phlorotannin isomers are listed. Isomers with [M-H]⁻ at *m/z* 373 (compound 1-3), phlorotannins trimers, were observed, which correspond tentatively to fucophloroethol. Isomers with [M-H]⁻ at *m/z* 497 (compound 4-5), phlorotannins tetramers, were observed, which correspond tentatively to fucodiphloroethol. Furthermore, isomers with [M-H]⁻ at *m/z* 745 (compound 9-11) and *m/z* 869 were tentatively identified as fucophloroethols with six or seven PGUs, respectively. Hydrogen migration was observed (noted as either +2 or -2 in Table 7.3) in some of the fragments. Even though the fragmentation patterns of the isomers showed some differences, indicating structural diversity, it was not possible to make further elucidation of the structures. Further structural identification of the isomers would require severe purification of the extracts as well as NMR.

Table 1. Elementary composition, retention time, UV (nm), [M-H]⁻, MS² [M-H]⁻ data and ECD responses (nAs) for compound (C) 1 to 13 from EAF

C	Elementary Composition	RT (min)	UV (nm)	[M-H]-	ppm	MS/MS fragmentation pattern	ECD [nAs]
1		3.256	205, 274sh	373.0564	-0.27	355.0448 (-H_2O), 329.0184, 311.0554, 305.0575, 287.0556, 269.0460, 243.0689, 229.0135 (-1PGU, - H_2O), 214.2045, 207.0282, 181.0493, 165.0192, 139.0370, 125.0244	-
2	$C_{18}H_{14}O_9$	7.048	210, 272sh	373.0560	-1.34	355.0459 (-H_2O), 329.0659, 305.0661, 287.0558, 261.0764, 243.0667, 231.0286 (-1PGU, -H_2O, +2), 216.0062, 205.0506, 189.0558, 165.0191, 149.0239, 141.0187, 124.0157 (-1)	63.10±1.54
3		9.324	213	373.0590	6.70	329.0381, 311.0579, 259.0246, 247.0243 (-1PGU), 229.0140 (-1PGU, -H_2O), 219.0287, 201.0190, 177.0211, 161.0223, 141.0191, 125.0248	-
4	$C_{24}H_{18}O_{12}$	8.335	208, 273sh	497.0729	0.80	479.0619 (-18), 453.0814, 435.0720, 413.0511, 395.0395, 371.0411 (-1PGU), 353.0307 (-1PGU, -18), 335.0200 (-1PGU, -2H_2O), 325.0353, 309.0404, 287.0191, 267.0305, 247.0242 (-2PGU, +2), 229.0145 (-2PGU, -H_2O, +2), 219.0299, 203.0348, 191.0346, 165.0190, 139.0032	5.63±0.59
5		8.471	210, 271sh	497.0717	-1.61	479.0612 (-H_2O), 453.0792, 435.0693, 411.0718, 395.0363, 371.0404 (-1PGU), 353.0292 (-PGU, -H_2O), 339.0500, 327.0507, 309.0390, 283.0250, 267.0305, 247.0242 (-2PGU, +2), 229.0133 (-2PGU, -H_2O, +2), 205.0496, 165.0199, 139.0031, 125.0234	
6	$C_{30}H_{22}O_{15}$	7.535	211, 275sh	621.0880	-0.97	603.0769 (-H_2O), 577.0974, 541.0765, 477.0423 (-1PGU, -H_2O), 455.0613, 433.0548, 413.0507, 373.0526 (-2PGU, +2), 343.0442, 311.0205, 287.0200, 247.0262, 207.0291 (-3PGUs, -2H_2O), 165.0189, 125.0241	24.63±1.54
7		9.708	208, 275sh	621.0891	0.81	603.0782 (-H_2O), 585.0654 (-2H_2O), 559.0864, 537.0660, 479.0607 (-1PGU, -H_2O, +2), 433.0539, 371.0400 (-2PGUs, +2), 353.0301 (-2PGUs, -H_2O, +2), 335.0194 (-2PGUs, -2H_2O, +2), 309.0401, 283.0233, 249.0403, 229.0140, 205.0503 (-3PGUs, -2H_2O, -2), 163.0404, 139.0034	2.52±0.16
8		9.964	212, 273sh	621.0879	-1.13	603.0778 (-H_2O), 585.0670 (-2H_2O), 559.0875, 519.0539, 477.0454 (-1PGU, -H_2O), 433.0565, 413.0302, 393.0224, 371.0398 (-2PGUs, +2), 339.0502, 309.0391, 283.0272, 245.0079 (-3PGUs, +2), 205.0492 (-3PGUs, -2H_2O, -2), 139.0029	1.32±0.18
9	$C_{36}H_{26}O_{18}$	9.423	212	745.1058	1.61	727.0949 (-H_2O), 701.1124, 659.0818, 579.0816, 537.0685, 477.0475 (-2PGU, -H_2O, +2), 411.0348, 355.0414, 311.0225 (-3PGU, -3H_2O, -2), 249.0416, 205.0158 (-4PGU, -2H_2O), 163.007	-
10		11.009	212, 272sh	745.1046	0.00	727.0935 (-H_2O), 665.0639, 619.0747 (-1PGU), 585.0676 (-1PGU, -2H_2O, +2), 559.0870, 517.0385, 477.0472 (-2PGU, -H_2O, +2), 441.0237, 389.0308, 353.0292, 309.0442, 231.0295, 205.0120 (-1PGU, -36), 177.0194, 139.0052	0.96±0.04
11		11.112	212	745.1049	0.40	727.0943 (-H_2O), 709.0858 (-2H_2O), 619.0735 (-1PGU), 583.0538 (-1PGU, -2 H_2O), 525.5692, 477.0478 (-2PGU, -18, +2), 443.0338, 371.0425, 339.0503, 263.0168, 229.0121, 203.0354 (-4PGU, -2H_2O, -2), 177.7941, 139.0033	
12	$C_{42}H_{30}O_{21}$	11.732	-	869.1238	3.57	851.1094 (-H_2O), 833.0920 (-2H_2O), 727.0922 (-1PGU, -H_2O, +2), 693.0811, 641.0496, 601.0574 (-2PGU, -H_2O, +2), 567.0528, 513.0421, 497.0697, 477.0392, 409.0238, 353.0272, 337.0401, 229.0136, 204.8425 (-5PGU, -2H_2O, +1), 139.0014	-
13		12.190	-	869.1198	-1.04	851.1086 (-H_2O), 775.0068, 744.0918 (-1PGU, +1), 689.0585, 619.0710 (-2PGU, +2), 583.0460 (-2PGU, -2H_2O, +2), 511.0480, 459.0351, 426.0419, 373.0522, 338.0352, 303.3885, 229.0143, 175.0369	-

On-line determination of antioxidant capacity of individual phlorotannins

Along the identification of phlorotannins in EAF, on-line detection of the antioxidant capacity of individual phlorotannins was carried out by HPLC-DAD-ECD. It was possible to detect compound 2 to 11 by UV (Fig. 7.6). It was possible to detect the antioxidant capacity of some individual phlorotannins and propose their contribution to the overall antioxidant capacity of EAF. However, for some compounds it was not possible to distinguish the ECD response, e.g. compound 3 and 9 have different composition, but could not be separated in the ECD, hence the ECD response of these two compounds were not determined. Non-separable ECD responses of compounds with the same composition were though determined. Hence, determination of antioxidant capacity of individual phlorotannins was only carried out for compound 2 to 11, excluding compound 3 and 9, by calculating the ECD response (nAs). The results are shown in Table 7.3.
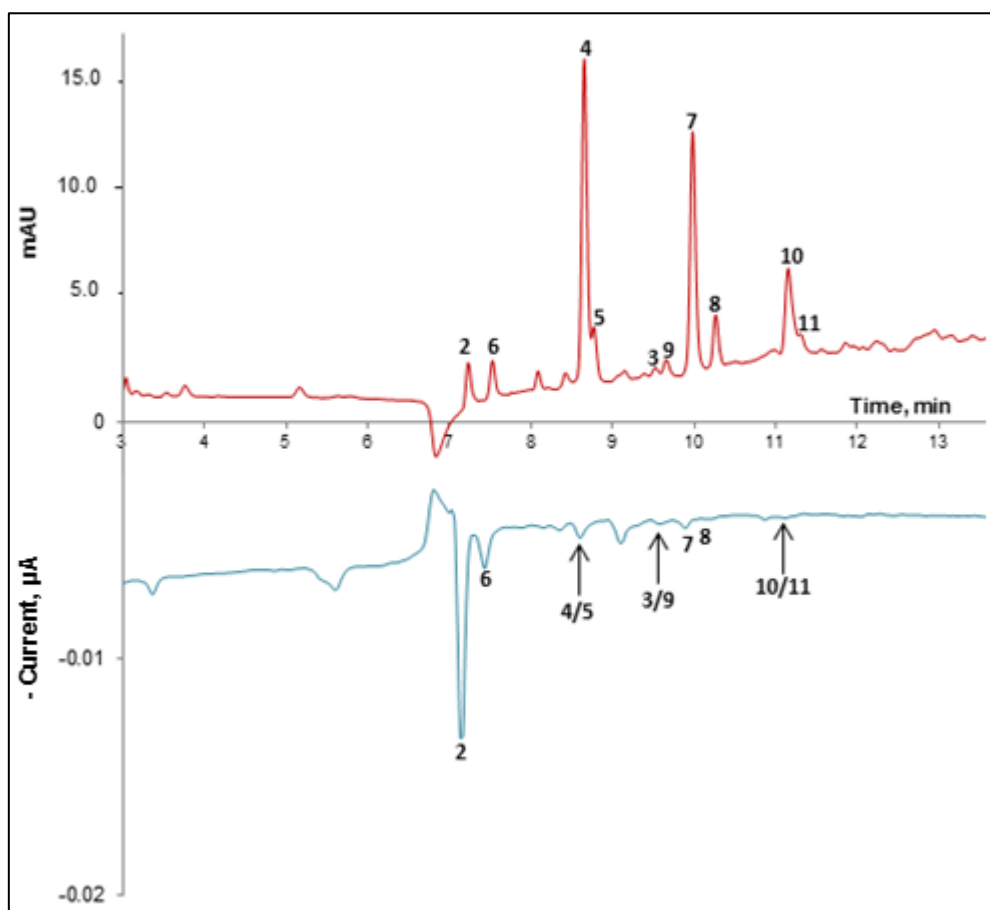
Fig. 7.6. UV chromatogram and amperogram corresponding to the HPLC-DAD-ECD analysis of EAF. The UV chromatogram at 280 nm (red) and amperogram (blue) in negative current, -µA. Compound 2 to 11 are numbered

The results indicate that Compound 2, an isomer of fucophloroethol (3 PGUs) has the highest antioxidant capacity. The antioxidant capacity seems to decrease with increased polymerization of phlorotannins. However, no significance difference between the areas was observed.

This study is the first of its kind to evaluate antioxidant capacity of individual phlorotannins. The structure dependant antioxidant capacity was discussed in section 5.6. The results on this area are contradicting. Some found a structure dependency similar to what was found in the present study. Others found no clear relationship between the antioxidant capacity (DPPH radical scavenging) and the degree of polymerization/size of phlorotannins. However, the antioxidant capacity of phlorotannins must be related to the available OH-groups. It can be hypothesised that large phlorotannin polymers might fold in a way which encloses the OH-groups inside the structure, and therefore poorer antioxidant capacity of large phlorotannins.

7.1.3. Evaluation of PLE for extraction of phlorotannins

Pressurised liquid extraction (PLE) was applied as a "green" extraction method. Water or 70% ethanol (v/v) were used as extraction solvents to obtain PLEW and PLEE, respectively. The phlorotannin extraction efficacy was evaluated by estimating the TPC and the antioxidant properties were determined by three antioxidant assays. The methods are described in Appendix A.2. The results are shown in Table 7.4.

Table 7.4. TPC (g GAE/100 mL extract) and antioxidant properties determined at different concentrations of PLEW and PLEE.

PLE extract	TPC	Antioxidant properties		
	g GAE/100 mL PLE extract	DPPH radical scavenging capacity* %	Iron chelating ability** %	Reducing power** OD_{700}
PLEW	2.4±1.3	49.2±1.2	18.8±7.2	0.2±0.0
		77.5±0.4	21.4±5.1	0.2±0.0
		83.7±3.1	17.8±4.7	0.3±0.0
PLEE	2.1±0.5	40.4±1.1	9.1±4.1	0.1±0.0
		71.7±1.2	7.5±0.4	0.3±0.2
		77.3±1.2	6.0±4.9	0.2±0.0

*Concentration 0.2, 0.6 and 1.0 mg PLE extract/mL water. **Concentration 1.0, 1.5 and 2.0 mg PLE extract/mL water

Extraction of phenolic compounds was successfully obtained with PLE. Water extracted slightly higher amounts of phenolic compounds compared with 70% ethanol. However the two extracts seemed to comprise very similar properties regarding antioxidant potential. PLEW showed slightly higher radical scavenging and iron chelating ability at the three concentrations used for the evaluation.

The PLE extracts were not tested in food and facial cream model systems due to limited access to extraction equipment to obtain adequate amounts for such studies. However, it would be interesting to find "green" adequate methods for extraction of phlorotannins from *F. vesiculosus* and this deserves more attention.

7.1.4. Summary - Part I

In summary, the two applied extraction methods obtained extracts containing phlorotannins. In solid liquid extraction (SLE) the phlorotannin yield depended on solvent polarity, as acetone and ethanol were more efficient in extracting phlorotannins compared with water. Furthermore, phlorotannin purification of 80% (v/v) ethanol extract was obtained by liquid-liquid partitioning (LLP) with ethyl acetate. Water extracts obtained from the young part (growing) of the seaweed showed higher phlorotannin content compared with extracts obtained from the old part (non-growing) of the seaweed. A high interfacial affinity of phlorotannins in especially AE, EE and EAF was observed in the partitioning study. This indicated a higher degree of extracted phlorotannins of an amphiphilic nature, which could increase the antioxidant efficacy of these compounds in emulsions as they are more likely to partition at the oil-water interface.

A high phenolic content of the SLE extracts and LLP fraction was associated with high antioxidant activity, especially radical scavenging and reducing power. Moderate iron chelating ability was observed for WE, AE and EE, with WE having the highest activity. Thus iron chelating ability was not positively correlated with high phlorotannin content. This indicated that phlorotannins might not contribute as much to this antioxidant property compared to other polar iron chelating compounds, e.g. polysaccharides, which might be co-extracted with water.

It was found that beside phlorotannins the SLE method mainly extracted tocopherols, pigments and iron. The composition was very much dependant on extraction solvent. The co-extracted pigments belonged to the major groups of carotenoids and chlorophylls. WoE and EE had the highest amount of both pigment groups. Fucoxanthin was the dominant carotenoid. The purification step of EE also seemed to concentrate the pigments, especially chlorophyll C2 and fucoxanthins (19-But-Fucoxanthin and fucoxanthin). Fucoxanthins, e.g. fucoxanthin, 19-B-fucoxanthin and 19-Hex-fucoxanthin, and lutein were correlated to the antioxidant properties. Hence, it could not be ruled out that these pigments influenced the antioxidant properties of the extracts. This thorough determination of co-extracted compounds provided new knowledge of how other compounds than phlorotannins contribute to the antioxidant activity of such extracts, both antioxidatively and prooxidatively. Hence, correlations between antioxidant properties and extracted compounds were obtained.

Tentative structural elucidation of 13 phlorotannin isomers from EAF was obtained by uHPLC-DAD-qTOFMS. It was not possible to determine the structural differences between isomers, though the fragmentation patterns obtained showed clear differences presumably due to different branching of the phlorotannins. Further structural identification may be obtained by a combination of NMR and tandem MS. However, this requires high purification of the phlorotannin extracts. On-line determination of antioxidant activity of the individual phlorotannins generally showed that low molecular phlorotannins exhibited higher antioxidant activity and also that the activity decreased with polymerisation. This method could be used as a fast screening of complex samples to identify the presence of highly antioxidative phlorotannins, e.g. isomers of fucophloroethol (3 PGUs).

Lastly, the tested alternative "green" pressurised liquid extraction (PLE) method successfully extracted phlorotannins using high temperature and high pressure. Water seemed to be slightly better in extracting phlorotannins under these conditions compared with 70% (v/v) ethanol, and water also obtained a PLE extract with high radical scavenging activity and moderate iron chelating ability similar to what was observed for SLE extracts obtained from ethanol and acetone. Hence, these preliminary results opens up for further evaluation of PLE extracts in emulsion systems.

7.2.   Part II: Application

This part aimed at evaluating the antioxidant efficacy of the SLE extract and LLP fraction in inhibiting lipid oxidation in FO-enriched foods and skin care model products, i.e. facial cream. The different factors influencing the efficacy will be discussed and correlations with results from Part I will be included.

7.2.1.   Antioxidant efficacy of Icelandic F. vesiculosus extracts in fish-oil-enriched foods

Five studies were conducted to evaluate the antioxidant efficacy of the SSL extracts and LLP fraction in tree types of FO-enriched food products; milk, mayonnaise and granola bars. The application of the extracts in the foods was outlined in section 6.2.1 and in Table 6.2. In the following the main findings on how the antioxidant acted regarding retarding lipid oxidation in the food systems are discussed.

In milk (Paper I)

WE and EAF were tested in 0.5% fish oil-enriched milk in three concentrations (1, 1.5 and 2 g dw/kg milk, low, middle and high, respectively). Antioxidant activity of both WE and EAF was found in milk emulsions, since an increased lag phase was observed for the formation of secondary oxidation products as illustrated by the development of 1-penten-3-ol in Fig. 7.7.
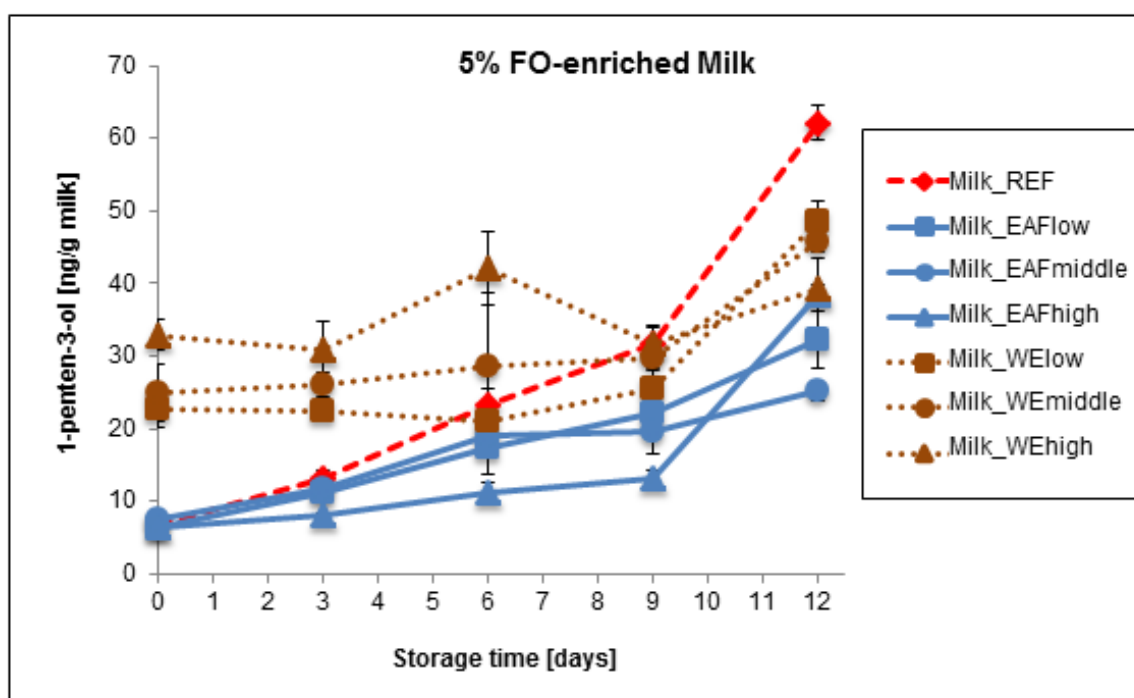
Fig. 7.7. Development of secondary volatile oxidation products (1-penten-3-ol) in 0.5% FO-enriched milk with WE (blue) or EAF (brown) in three concentrations (1 (low), 1.5 (middle), 2 (high) g dw/kg milk) including a control without added extract (Milk_REF, red) during 12 days of storage at 5°C (dark). Mean±SD (error bars)

Development of PV on the other hand was not decreased by addition of WE and EAF (results shown in Paper I). Generally, PV was higher for milk emulsions added WE and EAF until day 6, where after a decrease in PV was observed. The decrease observed after day 6 is because the conversion of primary oxidation products to secondary oxidation products was faster than the formation of primary oxidation products. Therefore it was expected that the formation of secondary oxidation products would also be high due to the higher oxidation offset in milk with WE or EAF added. This was not the case.

The higher observed formation of primary oxidation products compared with the control can be related to the physical properties of the milk and the added iron to the system from WE and EAF. Since milk has a low viscosity the diffusion of prooxidant transition metals, e.g. iron, is high which can increase their interactions with the oil phase and induce lipid oxidation. Moreover, the negatively charged surface of oil droplets in milk (described in section 3.5) might attract the positively charged transition metals from the water phase, hence again the iron will be more associated with the lipid surface. The results indicate that none of the extracts are strong iron chelators in the water phase, since ferrous ions are interacting with the lipids and initiating the lipid oxidation by formation of hydroperoxides. Hence, accumulation of hydroperoxides was observed. However, the decomposition of the accumulated hydroperoxides was inhibited by addition of

extracts, probably due to metal chelators which were able to hinder this decomposition and thereby the formation of secondary volatile oxidation products was limited.

Both WE and EAF contained tocopherols with potential radical scavenging capacity, which also gave an initially higher total tocopherol content (TTC) in milk with extracts added (seemed to be concentration dependant) (Fig. 7.8). A significant decrease in total tocopherol content (TTC) from day 0 to 6 of the milk without added extracts and milk added the lowest concentration of WE was observed. This indicated that either the extracts work as antioxidant and spare the tocopherols, or the extracts regenerate the tocopherols to work as antioxidants in the milk. Alemán et al. (2015) observed similar decrease in α-tocopherol concentration in FO-enriched milk emulsions without added antioxidants during storage.
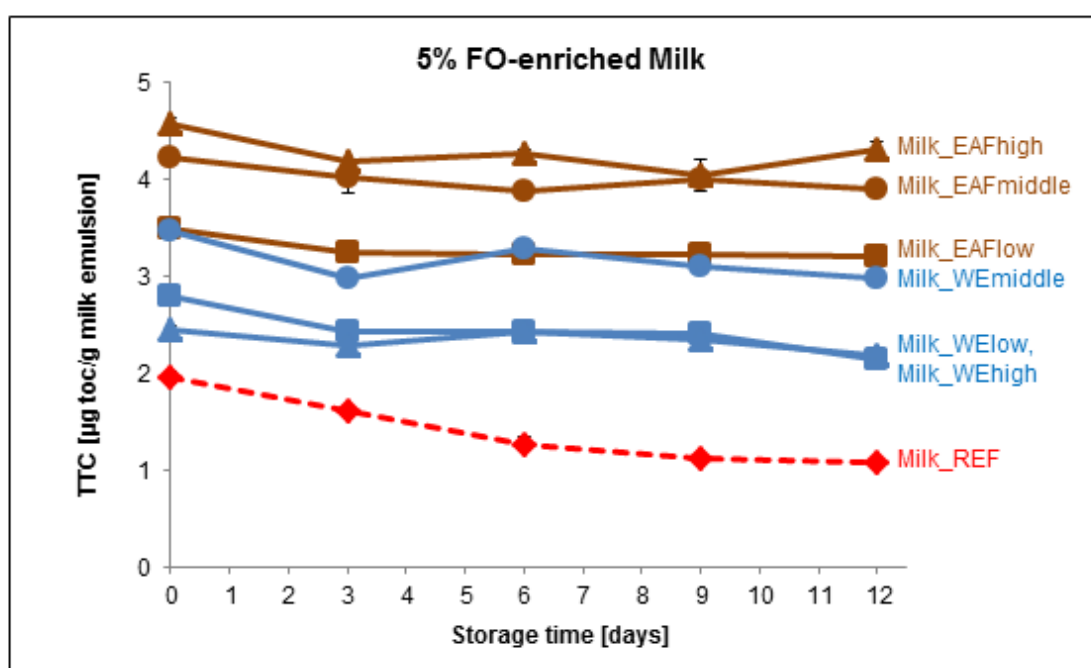
Fig. 7.8. Development in the total tocopherol content (TTC) in 0.5% FO-enriched milk with WE (blue) or EAF (brown) in three concentrations (1 (low), 1.5 (middle), 2 (high) g dw/kg milk) including a control without added extract (Milk_REF, red) during 12 days of storage at 5°C (dark). Mean±SD (error bars)

In mayonnaise (Paper I and II)

All the SLE extracts and the LLP fraction were tested in 15% FO-enriched mayonnaise. The study was divided in two, since WE and EAF were tested simultaneously with being tested in milk, and afterwards WoE, WyE, AE and EE were tested in mayonnaise alone. Concentrations between 1 and 2 g dw/kg mayonnaise (1.0 (low), 1.5 (middle) and 2.0 (high) g/kg) were added to the mayonnaise similar to what was added to the milk emulsions.

Antioxidant activity was observed for all the SLE extracts and the LLP fraction in FO-enriched mayonnaise. This antioxidant activity was observed as decreased oxidation of EPA and DHA, and increased lag phases of both PV and secondary volatile oxidation products. In Fig. 7.9 the development of 1-penten-3-ol, representing the development of secondary oxidation products in mayonnaise, is shown for all tested extracts, by combining results from the first and second mayonnaise study. The control mayonnaise without extract (Mayo_REF) in the Fig. 7.9 is the mean of the controls from the two studies.
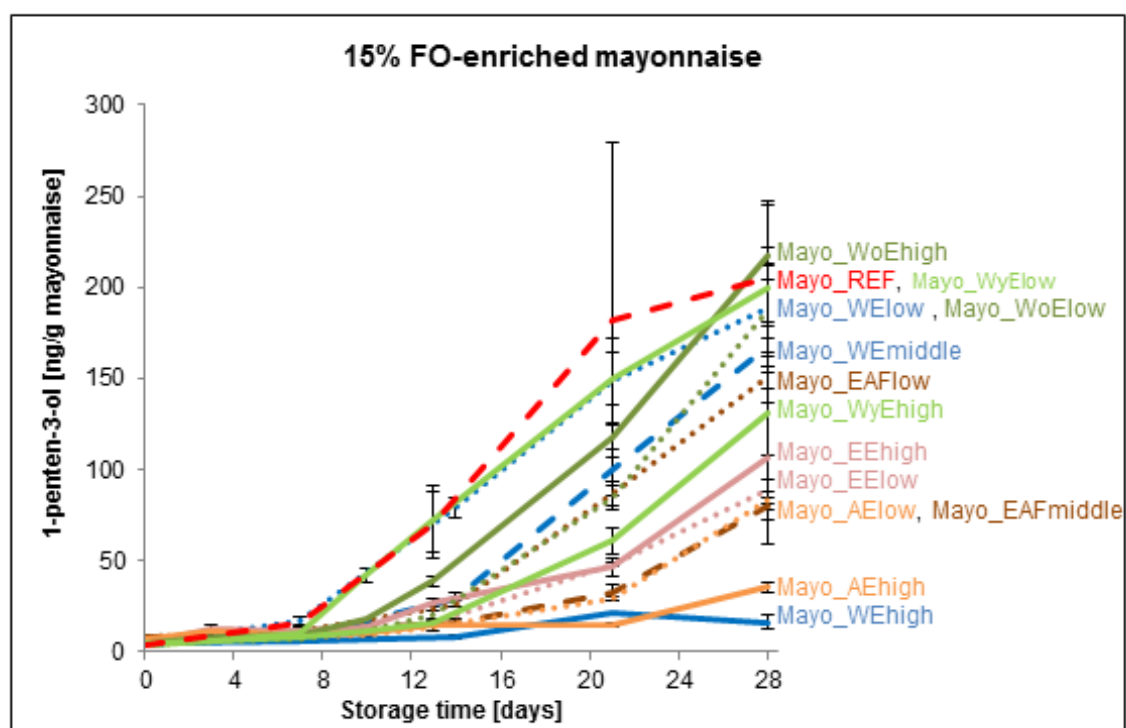
Fig. 7.9. Development of secondary volatile oxidation products (1-penten-3-ol) in15% FO-enriched mayonnaise with or without seaweed extract (WE, WoE, WyE, AE, EE or EAF) in two to three different concentrations (1 (low), 1.5 (middle) and 2 g (high) dw/kg mayonnaise) during 28 days of storage at 20°C (dark). Mean±SD (error bars)

The extracts can more or less be divided into two groups, depending on antioxidant efficacy; the water extracts (low efficacy) and the extracts obtained from acetone or ethanol, including the fraction (EAF) (high efficacy). However, one exception was that WE in the highest concentration was found to be very efficient and able to inhibit the formation of e.g. 1-penten-3-ol completely. A PCA of the second study, conducted to investigate the correlation between the extracts and the oxidation products during storage, showed a negative correlation of all oxidation products with AE, also indicating high antioxidant efficacy of this extract. Concentration dependent antioxidant efficacy was observed for all extracts in the mayonnaise. Beside WE, the second most efficient water extract was WyE when added to mayonnaise in the highest concentration, as this sample

was located in-between the two groups both in the development of PV and secondary oxidation products during storage (Fig. 7.9). Hence, the *F. vesiculosus* extract obtained from the young part of the seaweed seemed to be a more efficient antioxidant than the extract from the old part.

High iron chelating ability has been found to be a crucial antioxidant property in order for antioxidants to work efficiently in mayonnaise as discussed in section 4.3.3. Especially WE exhibited high iron chelating ability as shown in Table 7.1. However, the iron chelating ability of WE is related to co-extraction of other highly polar compounds other than phlorotannins, since no correlation between TPC and iron chelating ability was observed. EE and AE were also efficient antioxidants in the mayonnaise even though these extracts only exhibited moderate iron chelating ability. Thus, even though iron chelating ability seems to be an important property in FO-mayonnaise, high TPC and high radical scavenging capacity can make up for a lack in iron chelating ability, probably also due to the expected high affinity of phenolic compounds from AE to the oil/water interface (to work efficiently in emulsions) and thereby an efficient location of the antioxidants in the emulsions. However, very high radical scavenging capacity, as observed for EAF, could not make up for an almost non existing iron chelating ability, even though the phenolic compounds in EAF also had high affinity to the interface. This indicates that the extracts need to possess multifunctional properties, both high radical scavenging activity, moderate iron chelating ability and high interfacial interactions in order to work efficiently in mayonnaise.

Similar to what was observed in milk, a higher consumption of tocopherol was observed in the control mayonnaise without extract, however only in the first study of WE and EAF. The higher decrease of α-tocopherol in the control mayonnaise indicates that oxidation was more distinct in this sample and that α-tocopherol was used as antioxidant to a higher degree than in mayonnaise added WE or EAF. Thus, either the tocopherols are regenerated by the extracts or the extracts are used as antioxidants before tocopherol in mayonnaise with WE or EAF added.

In granola bars
Two granola bar trials were performed. In the first trial (Trial 1), WE, AE and EE were added in concentrations of 0.5 and 1.0 g/100 g FO emulsion. In the second trial (Trial 2) single layered (EM1) and multi layered (EM2) emulsions were produced to provide different surface charges of the emulsions. In the following the results of the two trials are presented and discussed.

Granola bars Trial 1 (Paper III)
Development of PV in the granola bars from trial 1 was somewhat different when seaweed extracts were added into the formulation compared to the control. In general granola bars added EE or AE in the low concentration, showed antioxidant activity as granola bars with these extracts added had

lower PVs during storage. On the other hand WE generally showed no effect on PV compared to the control. Moreover, addition of extracts increased the lag phase for 1-penten-3-ol, which was general for the secondary oxidation products (Fig. 7.10). Granola bars prepared with WE had a lag phase up to 4 weeks and hereafter the same development as the Control. Granola bars containing the high amount of EE had the longest lag phase for volatiles (up to 6 to 7 weeks) followed by granola bars with AE. Towards the end of storage the order of antioxidant efficacy to limit the formation of volatile secondary oxidation products in the bars was as follows: AE1 > EE2 > AE2 = EE1 = WE2 > WE1 > Control. The order was confirmed by a PCA model in Paper III, conducted on volatiles data from week 9. Preliminary sensory analysis of the granola bars was performed week 0, 5 and 10 (data not shown). It was clear that after 10 weeks of storage the fishy and rancid taste of the product had increased. However, it was observed that granola bars added EE and AE generally had lower fishy and rancid taste compared to the control without extract and those added WE.
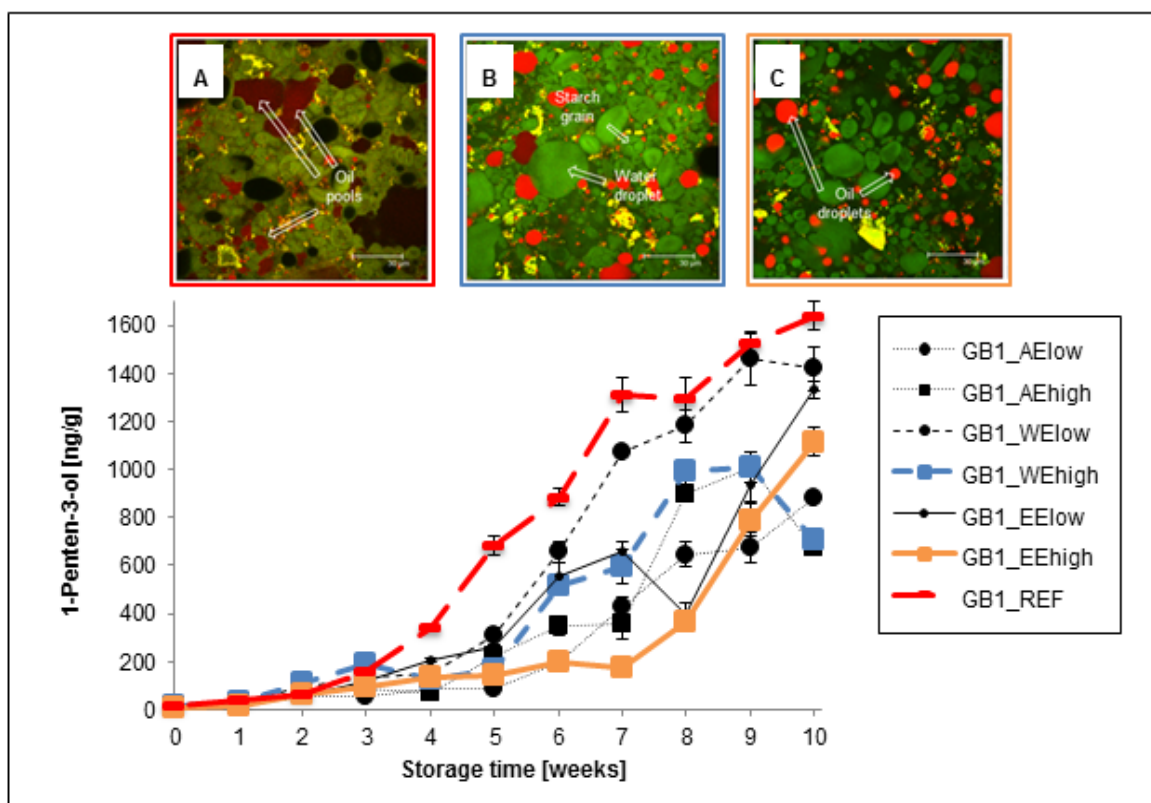
Fig. 7.10. Microstructure and development of secondary volatile oxidation products of the granola bars from Trial 1. Microstructure (CLSM images) of granola bars with or without extract (week 0 at 20°C), A/RED: The Control without extract. Development of 1-penten-3-ol (graphical), mean±SD (error bars) (n=3). B/BLUE: Granola bar added water extract (WE), C/ORANGE: Granola bar added ethanol extract (EE), all with 1% extract (high) in the FO emulsion. Scale bars are 30 μm.

The higher antioxidant efficacy of EE and AE was found to be related to the structure of the granola bars. Fig. 7.10 shows the structures revealed by confocal light scattering microscopy (CLSM). An efficient incorporation of the 70% FO emulsions into the granola bars was evaluated as intact oil droplets in the matrix. All CLMS images showed that oil was not only found as intact spherical oil droplets but also as large oil pools in the bars, which indicated ruptured droplets. The oil pools were most pronounced in the Control where no extract was added. Among samples with extracts, the oil pools were seen more often in granola bars prepared with WE, and more evenly distributed spherical oil droplets were observed in the bars prepared with EE and AE. This indicates that EE and AE have some emulsifying properties, which WE are lacking. It was expected that unprotected oil would be more prone to lipid oxidation since emulsification creates a protective structure around the oil droplets where also antioxidants could be located to hinder lipid oxidation.

The possible high interfacial affinity of antioxidative phenolic compounds in EE and AE compared to in WE would result in location of the phenolic compounds in both the water phase and the interface between oil and water. This will place the phenolic compounds in close contact to lipid oxidation initiators such as iron and also the radicals from the initial lipid oxidation in the oil phase. Radical scavenging capacity was found to be a more important property compared to iron chelating ability in low moisture foods such as crackers (Barden et al., 2015). Thus, Nielsen and Jacobsen (2009) also found that EDTA, an efficient iron chelator, increased oxidation in energy bars added emulsified FO compared to when no EDTA was present. Thus, the combination of high radical scavenging capacity and the expected efficient location of phenolic compounds might be the reason for the observed higher antioxidant efficiency of EE and AE in granola bars.

Consumption of α-, and γ-tocopherols was observed in all samples (Fig. 7.11). The fastest consumption of α-tocopherol was found in Control (marked with red in Fig. 7.11). The α-tocopherol reduction in Control reached 65% after only two weeks, whereas, at the same storage point no significant consumption of α-tocopherol was observed in granola bars with EE or AE, no matter the concentration. Horn et al. (2009) found that γ-tocopherol works efficiently as chain breaking antioxidants in energy bars added emulsified fish oil. Due to the lipophilic nature of tocopherols, they will, after emulsification, be located inside the oil droplets or at the oil-water interface and thereby close to the site of lipid oxidation. Phenolic compounds are able to regenerate tocopherol, it can therefore be assumed that extracts rich in amphiphilic phenolic compounds use their radical scavenging activity to regenerating tocopherols at the oil-water interface (Baldioli et al., 1996). Presumably, the regeneration of tocopherols gives an additional high antioxidant activity in the bars added extracts, especially EE and AE.
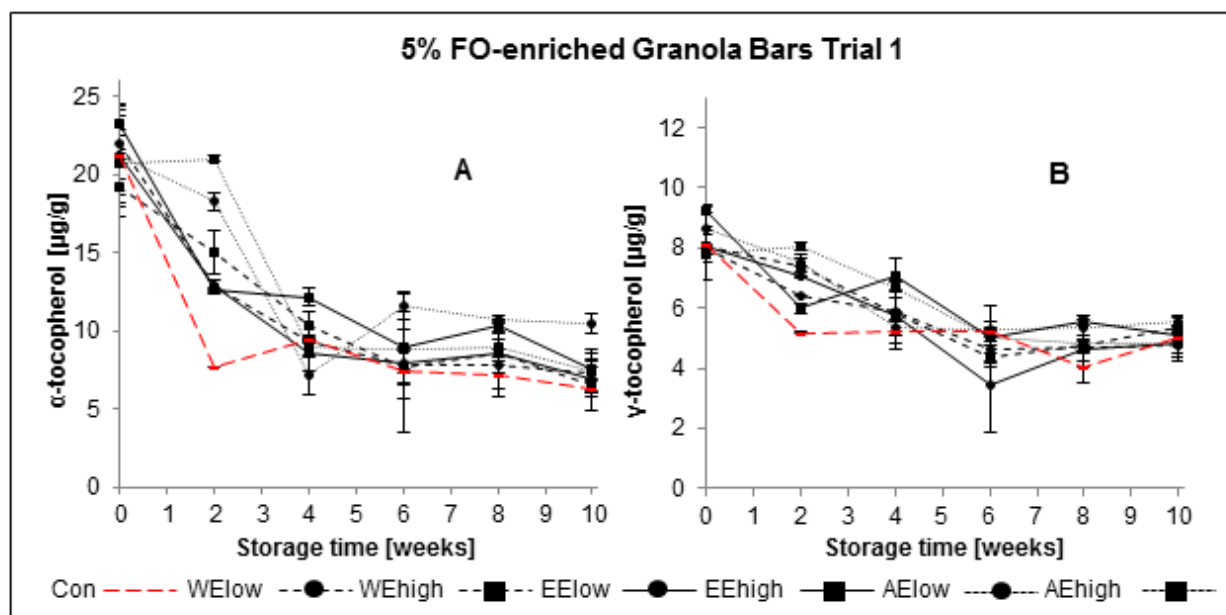
Fig. 7.11. Tocopherol content (µg/g granola bar). Development in concentrations (µg/g bar) of two tocopherol homologues: (α- (A) and γ-tocopherol (B)) in FO-enriched granola bars added F. vesiculosus water (WE), ethanol (EE) or acetone extract (AE) (0.5 (low) or 1% (high) in the FO emulsion) and a Control (Con, red) (without extract) stored dark for 10 weeks at room temperature (n=2). Mean±SD (error bars)

Granola bars Trial 2 (Paper IV)

In Trial 2 we aimed at optimising the interfacial layer around the oil droplets to provide an efficient barrier between prooxidants from the water phase, e.g. iron, and the oil phase. This was obtained by making 25% FO enriched primary (EM1) or secondary emulsions (EM2). The primary emulsions were produced with Na-cas as emulsifier, similar to what was used in Trial 1, resulting in a negatively charged surface of the oil droplets. The secondary emulsions were obtained by covering the negatively charged oil droplets from the primary emulsions with positively charged chitosan during homogenisation. The zeta-potential was measured and showed that the conversion from negatively to positively charged oil droplets was successful, as the initial charge of anionic droplets increased from -25 mV to approximately +21 mV with the addition of chitosan (0.5 w/v%). The emulsions obtained in combination with the *F. vesiculosus* extracts (WE, AE and EE) were evaluated in granola bars.
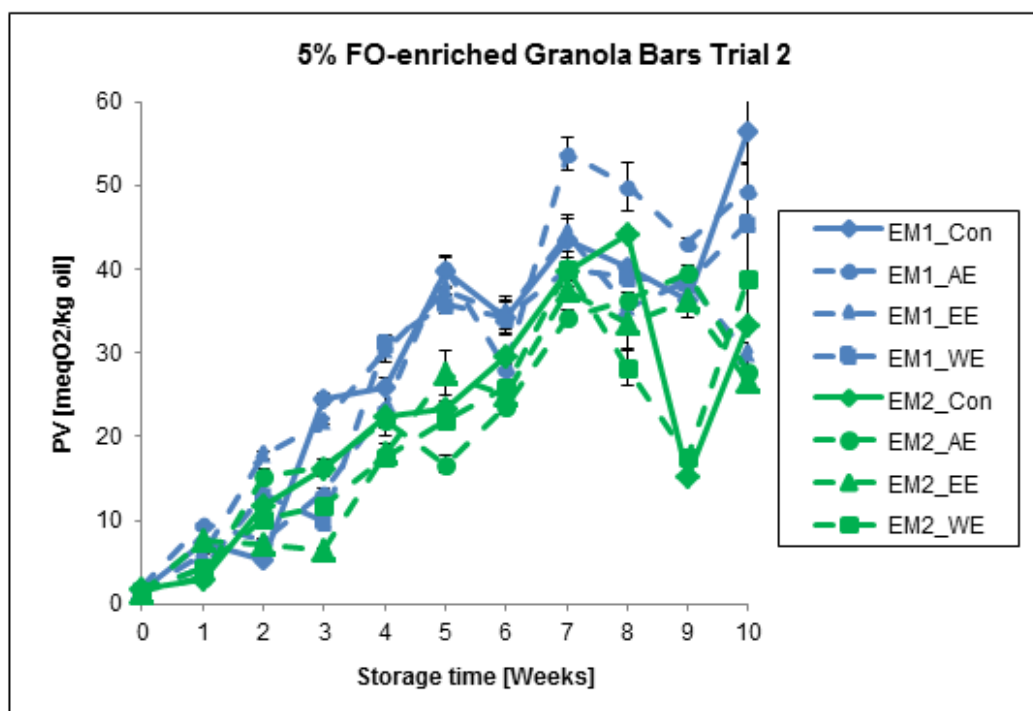
Fig. 7.12. Peroxide values (meq/kg oil) in granola bars enriched with fish oil emulsions (EM1: primary emulsion; EM2: secondary emulsions) containing acetone (AE), ethanol (EE) and water (WE) extract of F. vesiculosus, Control (Con, without extract) and stored dark for 10 weeks at room temperature (n=2). Mean±SD (error bars)

Among all granola bars, the samples prepared by secondary emulsions generally showed lower PV values compared to samples prepared by primary emulsions irrespective of whether seaweed extracts were added or not (Fig. 7.12). Klinkesorn (2005) found that FO/w emulsion droplets coated by lecithin and chitosan produce cationic droplets that are more stable towards oxidation than emulsions coated by anionic lecithin alone. They suggested that the positively charged droplets in the secondary emulsion inhibited iron-lipid interaction, presumably by decreasing the ability of iron to interact with the emulsion interface through electrostatic repulsion. They also suggested that the greater oxidation rates in primary anionic emulsions could be due to increased interfacial iron concentration because of attractive forces. The thickness of the interfacial layer is not only important to protect oil droplets against coalescing but may also protect lipids from oxidation by acting as a barrier to the penetration and diffusion of molecular species that promote oxidation.
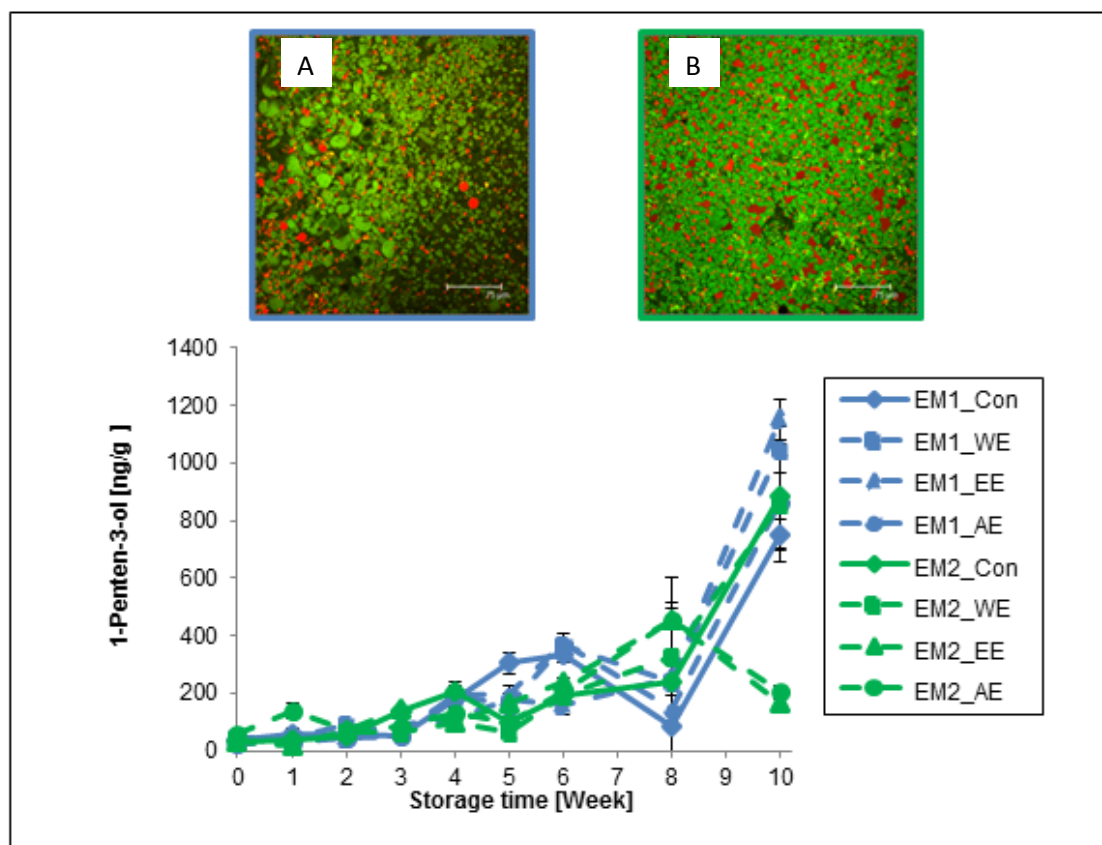
Fig. 7.13. Microstructure and development of secondary volatile oxidation products of granola bars from Trial 2. Microstructure (CLSM images) of granola bars added EE (week 0 at 20°C), A/blue: primary emulsion (EM1) and B/green: secondary emulsion (EM2). Scale bars are 75 µm. Development of 1-penten-3-ol (graphical), Blue: primary emulsion (EM1), Green: secondary emulsion (EM2), mean±SD (error bars) (n=3)

All volatile secondary oxidation products showed similar development patterns in volatile concentrations during the storage period, with a lag phase followed by rapid increase in the later part of the storage period, exemplified by the formation of 1-penten-3-ol (Fig. 7.13). In general the development among samples was the same until week 8, where the drastic increase in all primary emulsions and secondary emulsions without extracts and with WE was observed. No increase after week 8 was observed for secondary emulsion added EE and AE. Also, in a PCA model of all oxidation parameters (at week 10), the by far lowest observed content of volatiles and other oxidation parameters was found for secondary emulsions added AE and EE. Further studies are needed to investigate if this antioxidant effect of AE and EE would continue after week 10.

The confocal images revealed that secondary emulsions improved oil droplet incorporation significantly (images in Fig. 7.13). This also underlines the results of the development in PV, and the fact that second emulsions are both more oxidatively and physically stable. The fact that no increase in secondary oxidation products in secondary emulsions added EE and AE were

observed after week 8, indicated a combination effect of EE and AE with the secondary emulsion with a positive influence on the oxidative stability.

7.2.2. *Antioxidant efficacy of Icelandic F. vesiculosus extracts in skin care products*

The antioxidant efficacy of WE and AE was evaluated in facial cream to evaluate the possible application in this type of system. Other issues are important in facial creams compared to foods, e.g. storage conditions can be more extreme. Hence, the possible antioxidant activity of the extracts against photo- or thermooxidation was evaluated in relation to cream stored dark and at ambient temperatures.

Facial cream model (Paper V)

The oxidative stability of facial cream added WE and AE stored under control (dark, room temperatures (A0)) or accelerated conditions (exposure to light (A+) or elevated temperatures (H0)) was increased by addition of extracts. This was mainly observed as an antioxidant activity of the extracts towards formation of secondary oxidation products. However, undesirable physical changes in the products were observed when the extracts were added.

The accelerated storage conditions induced some degree of emulsion disrupture during storage. The largest instability was observed in emulsions stored at high temperature (H0, storage in the dark at 40°C), which caused a large destabilization of the emulsions, leading to an evident syneresis at the end of the storage (visual evaluation). The largest increase in oil droplet size, and thereby instability of the emulsions, was observed when the high concentration of WE and AE was added no matter the storage condition. Moreover, visible colour development during storage was observed in all creams added extracts compared to the control (Fig. 7.14). The most pronounced colour development was observed for facial cream added the highest concentration of AE and stored in the dark at high temperatures. These colour differences pointed out that the use of seaweed extracts did not perfectly mimic the characteristics of conventional skin care emulsions, due to the presence of pigments in the extracts. The storage conditions could induce oxidative reactions that might affect pigments such as fucuxanthin and chlorophylls and produce colour changes in the creams. However, this deserves more research.
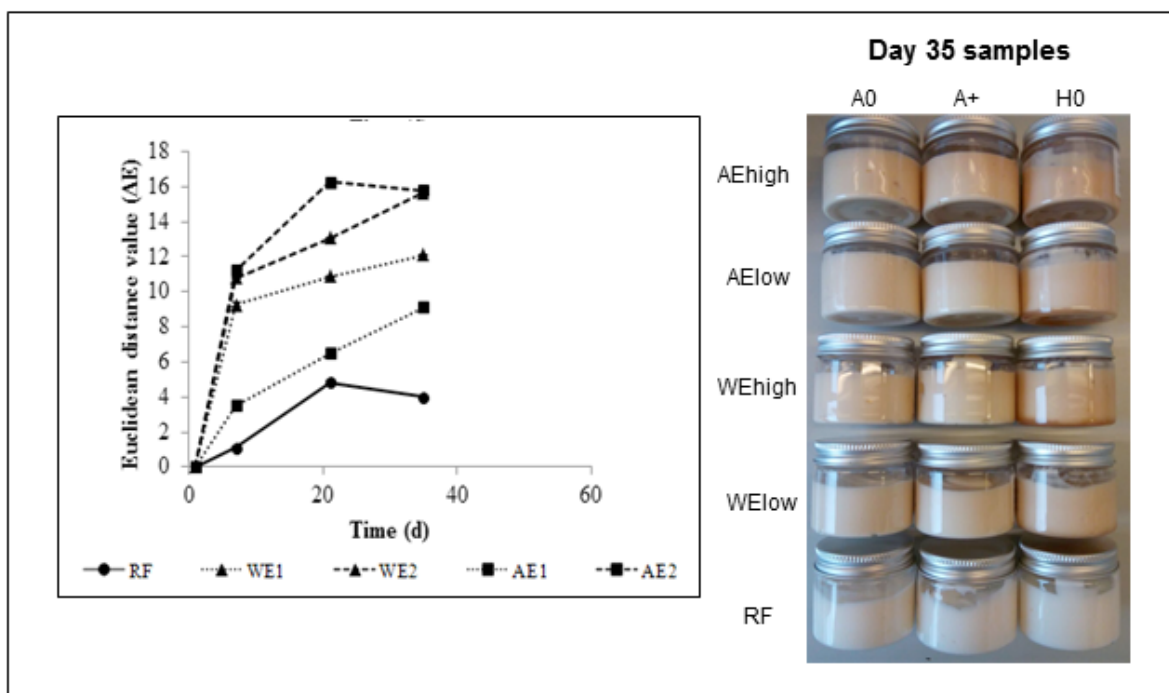
Fig. 7.14. Euclidean distance value of the cosmetic emulsions calculated during storage at high temperatures (H0) (graph) and images of the creams where the colour difference is valuable. A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1/WElow, water extract (low conc., 1 mg/g); WE2/WEhigh, water extract (high conc., 2 mg/g); AE1/AElow, acetone extract (low conc., 1 mg/g); AE2/AEhigh, acetone extract (high conc., 2 mg/g). Error bars indicate SD of the measurements

PV results from this study are stated in Paper V. In Fig. 7.15, the development of pentanal and heptanal is representing the groups of secondary oxidation compounds resulting mainly from the autooxidation of oleic, linoleic and α-linolenic acid (Belitz et al., 2009; Guillen and Uriarte, 2012; Poyato et al., 2014).

Generally, at the beginning of the storage, facial creams added high concentrations of extract (WE and AE) showed slightly higher PV values than facial cream without extract ($p < 0.05$). This could be a consequence of the presence of trace metals in the extracts (Table 7.2) which promoted, together with the temperature of processing (70-75 °C), oxidative reactions at an initial stage. During storage significant increase ($p < 0.05$) of PV were found in all samples. The high accumulation of PV in the beginning of storage also resulted in a higher initial concentration of secondary oxidation products in facial creams added extract.
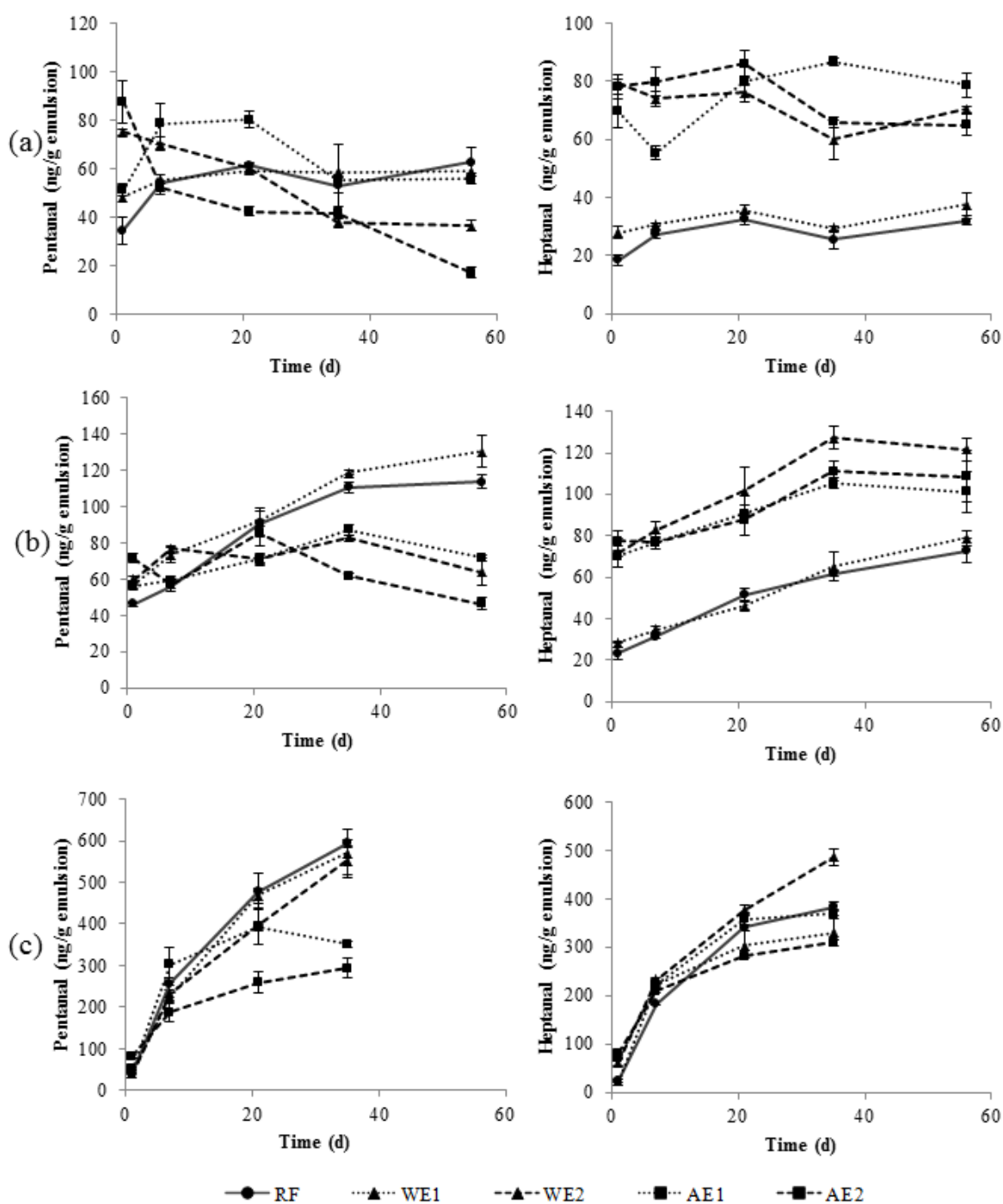
Fig. 7.15. Development of volatile compounds, pentanal and heptanal (ng/g emulsion), during storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements

In facial creams added WE stored in light (A+) at the end of storage had lower PV than facial cream without extract ($p < 0.05$). This could be due to the high content of carotenoids in WE (Table 7.2), as carotenoids are well known inhibitors of free radical chain reactions caused by photooxidation process (see section 4.4.2). Facial cream stored at high temperatures (H0) generally was more oxidised compared to facial creams stored at other conditions (A0 and A+) both regarding development of PV and secondary volatile oxidation products. A higher oxidation was though found in the facial cream without extract compared to those added extract, indication antioxidant activity of the WE and AE.

In the facial creams with the highest extract content (2 g/kg cream) pentanal showed significantly lower concentrations compared to facial cream without extract at the end of the storage at all storage conditions. On the other hand, the amount of heptanal at the end of storage, was significantly higher in facial creams added the high concentration of extract compared to facial cream without extract, except when stored at high temperatures. Thus, even though the presence of extract at the beginning of the storage resulted in higher amounts of pentanal and heptanal in all facial creams, lower oxidation rates were observed during storage in these creams. This indicates an antioxidant activity of the extracts in facial creams towards formation of pentanal and heptanal.

WE was generally more efficient in inhibiting lipid oxidation in facial creams. Iron chelating ability was thus found to be the most important antioxidant property in the creams stored dark, whereas in creams exposed to photooxidation (A+) both the presence of carotenoids and iron chelating ability was important the antioxidant activity of this extract. Antioxidant activity of AE in facial creams was either the same or slightly lower compared to WE at all storage conditions. Thus, the combination of high radical scavenging of phlorotannins in AE and the moderate iron chelating ability seems to make up for lack of iron chelating ability, as discussed above for mayonnaise (section 7.2.1). The iron chelators form complexes with metals and inactivate their catalytic effects in promoting lipid hydroperoxide decomposition. Due to this antioxidant effect, a general accumulation of primary oxidation products were observed in the extract containing facial creams and consequently lead to a lower formation of secondary volatile oxidation compounds. Moreover, a decrease in total tocopherol content (TTC) was observed in all samples during storage, and the highest rate of decrease was observed at the high temperature conditions (H0). It is worthy to highlight that the AE showed the highest tocopherol protective effect at all storage conditions, with AE2 being the best concentration. This indicates a similar trend as observed in e.g. the granola bars where phlorotannins possibly regenerated tocopherols as well as they worked as radical scavengers.

7.2.3. Summary - Part II

Testing of the SLE extracts and the LLP fraction in FO-enriched foods and facial cream model demonstrated their potential application as natural antioxidants. It has previously been demonstrated how challenging antioxidant applications in complex systems can be, and that it is difficult to extrapolate results from one system to another. In Table 7.5 the antioxidant efficacy of WE, AE and EE is summarized. WE, AE and EE were tested in most products and the table helps visualising how the efficacy between test products and extract types are different.

The test products had very different composition and previous studies have shown how antioxidant efficiency in these different products requires very specific antioxidant properties (c.f. section 4.3). In Part I we found that the antioxidant properties of the extracts depended on the composition of the extracts, and how the phlorotannin content was positively correlated with radical scavenging activity and reducing power. Iron chelating ability was not correlated directly with the phlorotannin content but more towards the presence of 19-hex-fucoxanthin.

In FO-enriched milk and mayonnaise, iron chelating ability has been found to be an important antioxidant property, since iron in the water phase interacts with lipids at the oil/water interface to initiate lipid oxidation or induce the break-down of lipid hydroperoxides to secondary oxidation products. The importance of iron chelating ability was also found particularly in the mayonnaise, since the water extract (WE) with the highest iron chelating ability was found to hinder formation of secondary oxidation products to the largest extent. This is seen as a strong antioxidant activity (> 50%) of WE in Table 7.5 towards formation of secondary oxidation products, but not on formation of primary oxidation products, where it actually had a prooxidant effect (< -5%), probably due to its ability to prevent decomposition of peroxides. WoE and WyE (water extracts obtained from the old or the young part of the seaweed, respectively) exhibited both low radical scavenging activity and low iron chelating ability, thus very poor antioxidant efficacy was observed for these two extracts in mayonnaise. However, high radical scavenging capacity and reducing power due to high phlorotannin content in the extracts in combination with moderate iron chelating ability also showed efficiency towards limiting lipid oxidation in milk and mayonnaise. This is seen for AE and EE in Table 7.5., as these extracts both showed intermediate (20-50%) to strong antioxidant activity in the mayonnaise for both formation of primary and secondary oxidation products. The antioxidant mechanisms of the phlorotannins in these types of products can be assumed mainly to be chain breaking free radical scavengers to hinder formation primary oxidation products or to regenerate tocopherols?. Therefore we observe this antioxidant activity of extracts with only moderate iron chelating ability, as long as they have high radical scavenging activity. The recommended extracts

in FO-enriched milk and mayonnaise among those we have tested is WE, AE (acetone extract) and EE (ethanol extract) in a concentration of 2 g dw/kg product.

In granola bars from Trial 1, AE and EE, prevented oxidation to a higher degree compared with WE, which generally showed no or low antioxidant activity in this product, according to table 7.5. In this first trial the antioxidant efficacy was associated with improved incorporation of FO preemulsions into the dough when AE and EE were added as observed by microscopic imaging. In the partitioning study we found that phenolic compounds in AE and EE to a larger extent were located at the interface indicating extraction of more amphiphilic phlorotannins with either acetone or water compared to what was extracted with water. Hence, in a system like granola bars the partitioning of the phlorotannins at the surface of the oil droplet possibly made them able to work as chain breaking antioxidant by deactivating lipid peroxyl radicals present here. It can also be suggested that the amphiphilic phlorotannins regenerated antioxidative tocopherols at the interface, as we also observed a decrease in the consumption rate of tocopherols in granola bars from Trial 1.

The oxidative stability of granola bars added either single emulsified fish oil emulsions or multi-layer fish oil emulsions to granola bars was studied in a second trial, in order to evaluate how negatively or positively charged oil droplets affected the oxidative stability of this type of product. It was found that additional protection against lipid oxidation was obtained when multilayer fish oil emulsions were added to the granola bars especially in combination with AE and EE (Table 7.5). The results from these studies clearly indicated the importance of the presence of high levels of phlorotannins in the extracts, thus AE and EE can be recommended for application in these types of low moisture product, even at the low concentrations tested (0.4 g dw/kg product)

In facial cream the oxidative stability was improved by addition of WE and AE, observed as a decreased formation of secondary oxidation products as it also is seen in Table 7.5. Thus, iron chelating ability was found to be an important antioxidant property in the facial creams stored dark, whereas it was suggested that for facial creams stored in light, the combination of iron chelating ability and presence of quenching carotenoids protected the facial creams against photooxidation. Even though, the oxidative stability was improved by addition of extracts, the physical stability of the facial creams were jeopardised when the extracts were added. Facial creams stored under extreme conditions (40°C or light) was destabilised and in some of the sample even syneresis was observed after only 35 days of storage. Moreover, an undesired browning of creams added extracts was observed, even in the control creams stored in the dark at room temperature. These undesirable quality changes in the facial creams added seaweed extracts can be related to the high content of pigments in the extracts. The storage conditions could induce oxidative reactions

that might affect pigments such as fucuxanthin and chlorophylls and produce colour changes in the creams. Removal of pigments from the extracts could possibly solve this problem. However, the observed antioxidant activity of e.g. WE, regarding photooxidation could then be lost.

Table 7.5. Ability of WE, AE and EE to inhibit lipid oxidation (Primary (PV) and secondary volatile oxidation products (Vol)) in different FO-enriched food products and in facial cream model.

Product	Concen-tration [g dw/kg product]	WE		AE		EE	
		PV	Vol*	PV	Vol*	PV	Vol*
Milk 1.5% fat 0.5% FO	1.0	-44	53	n.t.	n.t.	n.t.	n.t.
	1.5	-99	68	n.t.	n.t.	n.t.	n.t.
	2.0	-57	42	n.t.	n.t.	n.t.	n.t.
Mayonnaise 80% fat 15% FO	1.0	-7	26	20	50	30	45
	1.5	-18	34	n.t.	n.t.	n.t.	n.t.
	2.0	73	95	45	82	31	34
Granola bars Trial 1, 5% FO**	0.4	-1	4	9	56	6	39
	0.7	1	34	9	34	2	48
Granola bars Trial 2, EM1, 5% FO	0.4	20	-40	14	-18	49	-57
Granola bars Trial 2, EM2, 5% FO	0.4	32	3	54	83	54	84
Facial cream model — A0	1.0	16	47	-129	31	n.t.	n.t.
	2.0	-86	325	-45	431	n.t.	n.t.
A+	1.0	5	-2	47	37	n.t.	n.t.
	2.0	-3	0	-41	37	n.t.	n.t.
H0***	1.0	4	14	-7	17	n.t.	n.t.
	2.0	22	-18	22	36	n.t.	n.t.

*1-penten-3-ol in FO-enriched food products and heptanal in facial cream model, **end value at week 9, ***end value at day 35

n.t. = Product where the extract has not been tested*. Negative value indicates prooxidative effect and positive value indicates antioxidative effect

7.3.   References

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Chapter 8: Conclusions and Perspectives

8.1. Conclusion

The main objective of this PhD work was to evaluate the antioxidant efficacy of Icelandic *F. vesiculosus* extracts in real food and skin care products and outline the influence of antioxidant properties and composition on the efficacy. Furthermore the aim was to elucidate the antioxidant properties of phlorotannins and their contribution to the overall antioxidant activity.

Regarding the extraction methods, it was initially hypothesised that different extraction methods can be applied to extract phlorotannins from *F. vesiculosus,* and that the extraction methods applied affect the composition and antioxidant activity of such extracts. This hypothesis was confirmed as both solid-liquid extraction (SLE) and pressurized liquid extraction (PLE) successfully extracted phlorotannins. Various solvents were used to further evaluate the phlorotannin extraction efficacy of the two extraction methods applied. Whereas, ethanol and acetone was found to extract the highest amounts of phlorotannins by SLE, water was most efficient for extraction of phlorotannins by PLE. SLE was shown to extract a variety of other compounds than phlorotannins, especially tocopherols, pigments and iron. The composition of co-extracted compounds was very much dependant on the solvent. Thus, water to a higher degree extracted tocopherols and iron, and acetone and ethanol to a larger extent pigments.

We hypothesized that even though both antioxidants and prooxidants will be co-extracted, phlorotannins will be the main contributors to the antioxidant activity of *F. vesiculosus* extracts. A PCA analysis revealed that there were strong positive correlations between phlorotannin content, DPPH radical scavenging activity and reducing power of various seaweed extracts, underlining the importance of phlorotannins regarding the antioxidant properties. On the contrary, iron chelating ability correlated neither with the phlorotannin content, nor with the radical scavenging activity or reducing power. The PCA analysis furthermore indicated positive correlation between concentrations of pigments, e.g. fucoxanthin and lutein, and either iron chelating ability or radical scavenging activity and reducing power. Thus, it cannot be ruled out that these pigments contributed to the antioxidant properties of the extracts. These studies clearly demonstrated that phlorotannins from *F. vesiculosus* mainly act as potent free radical scavengers and primary chain breaking antioxidants. The phlorotannins do not appear to be efficient iron chelators.

A phlorotannin-enriched fraction was obtained by liquid-liquid partitioning (LLP) with ethyl acetate of the ethanolic SLE extract. This LLP fraction showed much higher radical scavenging activity and reducing power than the crude extracts. Thus, the LLP fraction was used to confirm the hypothesis regarding how different phlorotannins contribute to the overall antioxidant activity of the extracts

due to structural differences. Hence, structural elucidation of phlorotannins revealed the presence of 13 phlorotannin isomers, mainly relatively low-molecular weight phlorotannins, in the range from 374 to 870 Da. It was possible to determine the antioxidant activity of the individual phlorotannin isomers by an on-line method. The antioxidant activity seemed to decrease with increased polymerization. This study is the first of its kind on seaweed extracts to demonstrate that this method can be a future useful tool for fast screening of the presence of antioxidative phlorotannins in seaweed extracts.

It was hypothesised that *F. vesiculosus* extracts contain a wide range of antioxidants making them multifunctional, and thereby capable of performing efficiently in different food and skin care products. This was confirmed by testing the SLE extracts and the phlorotannin-enriched fraction in FO-enriched foods and one facial cream model. The test products had very different composition and previous studies have shown how antioxidant efficiency in these different products calls for very specific antioxidant properties, e.g. iron chelating ability in FO-enriched milk and mayonnaise, and radical scavenging activity in FO-enriched granola bars. The multifunctional nature of the extracts was yet found to be beneficial, since antioxidant activity was observed in all tested systems. The efficacy of the extracts was found to be both product and concentration dependant. In FO-enriched milk and mayonnaise, the tested extracts and the phlorotannin-enriched fraction was found to hinder formation of secondary oxidation products, and in some cases also the formation of primary oxidation products. The water extract (WE) obtained from the whole seaweed plant showed, when added in a concentration of 2 g dw/kg product, the highest antioxidant activity compared with the other crude extracts. Whereas WE generally showed high antioxidant activity in mayonnaise, the water extract obtained from the old non-growing part (WoE) of the *F. vesiculosus* plant, showed poor or no activity in mayonnaise. The water extract obtained from the young growing part (WyE) of the seaweed showed antioxidant activity, though not as high as WE. In granola bars, the acetone and ethanol extracts prevented oxidation to a higher degree compared with the water extract. The oxidative stability of the granola bars were further improved by the combination of acetone and ethanol extracts with multi-layer emulsions with chitosan. In accelerated studies of water and acetone extracts added to facial cream, the water extract showed slight antioxidant activity against photooxidation, whereas the acetone extract showed no antioxidant activity in this type of system.

The specific mechanism by which the phlorotannins prevent lipid oxidation can only be hypothesised. Several explanations have been put forward in this PhD work in order to explain the diverse antioxidant efficacy between extracts and type of test product. It was clear that phlorotannins might not always play the major role against lipid oxidation in these products, since

in some cases the antioxidant properties of the extracts are more important than the presence of phlorotannins. Granola bars yet remains an excellent example of phlorotannins performing in a product. The partitioning study revealed that extraction with solvents like ethanol and acetone possibly extracted more amphiphilic phlorotannins with high affinity to the interface between water and oil in w/o emulsions. Hence, the partitioning of the phlorotannins at the surface of the oil droplet possibly made them able to work as chain breaking antioxidant by deactivating lipid peroxyl radicals present here. We further suggested that the potency of amphiphilic phlorotannins to regenerate the endogenous antioxidant α-tocopherol could be another possible mechanism in this product.

The studies provide a necessary basis for, and more importantly will hopefully stimulate future development of natural antioxidants derived from seaweed for the food and cosmetic industry. The results also suggested the potential application of seaweed extracts rich in phlorotannins in the formulation of functional foods.

8.2. Future perspectives

Marine algae are an attractive source of antioxidants, which until now has been highly underutilized for development of antioxidant ingredients for the food and cosmetic industry.

In order to apply seaweed based antioxidants to different food and cosmetic products for human consumption, the extraction techniques are of extreme importance. Solvent extraction was in the present study shown to be effective when considering phlorotannins from *F. vesiculosus*. However, the solvents must also be adequately selected to meet requirements set by the EU regulations. Due to increased consumer awareness and demands for natural products, it is necessary to develop efficient and environmental friendly extraction techniques when retrieving antioxidants from algal material. Pressurized liquid extraction, where water or "green" solvents like ethanol can be used as co-solvent in extraction of phlorotannins, is as documented here an environmental friendly technique with good potential. The results from the present study are preliminary, but show efficient extraction of phlorotannins resulting in extracts with high radical scavenging activity and moderate iron chelating ability. Other "green" extraction methods like supercritical fluid extraction (SFE) could also be tested for this purpose. Furthermore, up-scaling of the extraction process should be considered to a future production of natural antioxidant from *F. vesiculosus*.

The current study showed that extraction of phlorotannins also led to extraction of a wide range of co-extracted compounds. Since the study only considered the influence of co-extracted compounds on the antioxidant activity of *F. vesiculosus* extracts, the presence of possible hazardous compounds was not taken into account. Previous studies have detected e.g. inorganic

arsenic in seaweeds. Inorganic arsenic can increase the risk of lung cancers even at very low intake. Moreover, seaweeds can take up heavy metals from ambient waters, such as iodine and cadmium. A high human intake of cadmium over many years can cause kidney damage. Seaweed has also been shown to contain natural marine toxins such as kainic acid and domoic acid, where kainic acid is a potent neuroexcitatory amino acid, though kainic acid has only been detected in the red algae *Palmaria palmata* (dulse).

A sustainable *F. vesiculosus* cultivation and harvesting protocol is needed in order to meet future demands for natural antioxidants derived from this seaweed. It is known that *F. vesiculosus* grows slowly and does not produce that much biomass during a season. However the phlorotannin content is more concentrated in *F. vesiculosus*, thus less biomass is in turn needed to obtain *F. vesiculosus* extracts with high phlorotannin content.

Optimal growth conditions for *F. vesiculosus* encompass salinities of at least 4-5 PSU, cold water conditions (<18°C), an adequate light regime (at least 8 hours per day), accessible nitrogen, and a suitable substrate for attachment in a sheltered or moderately exposed area. In order to most efficiently meet these growth requirements onshore cultivation in tanks could be a future option for growing *F. vesiculosus*. However, tank cultivation is costly, manpower demanding, and it takes up valuable space on land. It is therefore not likely that the immediate future of *F. vesiculosus* cultivation will take place along this high technology track. Offshore cultivation in one form or the other is therefore a more likely immediate possibility. The Danish Belt Sea and the Kattegat area meet some of the growth requirements of *F. vesiculosus* such as medium salinity (app. 20 PSU), cold water and moderately exposed intertidal areas. Cultivation trials of creating optimal conditions for vegetative growth of *F. vesiculosus* have been carried out in Denmark (coast of Endelave). Here, nets on the bottom of the sea positioned next to a natural population of *F. vesiculosus* significantly increased the attachment of young germlings within the area leading to an overall enhancement of biomass production. This method could when properly scaled result in annual production figures that might interest relevant industrial sectors. A general problem with offshore cultivation is that the environmental conditions are beyond control, which can result in large variations in the product and its composition.

Another approach could be to combine onshore and offshore cultivation. The seeding (on lines or nets) and initial growth could be carried out in tanks to provide the optimal conditions for the young seaweed plant to grow into a strong individual before being transferred for on-growth in the natural environment. It has been stated that the early stage of growth is essential to the development and well-being of the adult. Also, controlling the environmental conditions during this early phase, such as light, can decrease the attachment of epiphytes.

It has been stated that *F. vesiculosus* has a pronounced seasonal variation in phlorotannin content. The phlorotannin content thus reaches it maximum in the summer and early autumn due to the UV radiation defence mechanism of phlorotannins in brown algae. Therefore, for utilization of *F. vesiculosus* for antioxidant purposes, the seaweed should be harvested at this time a year (August or September) using a gentle harvesting concept that does leave the plant attached and capable of further growth.

The overall environmental impact should be considered prior to the start-up of cultivation trials involving *F. vesiculosus*. A positive aspect here is the fact that an increase in intertidal algal growth will be ecologically sustainable and beneficial in the context of marine biodiversity at large through the increase in marine micro habitats. More research is needed on the possible hazardous compounds which can be co-extracted when aiming at extracting high amounts of phlorotannins. Moreover, cultivation trials should be done for *F. vesiculosus* to elucidate the cultivation possibilities to obtain a sustainable production suited to meet the demands for natural antioxidants for the food and cosmetic industry.

8.3. Links

http://www.dr.dk/nyheder/regionale/midtvest/faa-dansk-tang-paa-middagsbordet

http://videnskab.dk/kort-nyt/forskningsprojekt-tang-pa-middagsbordet

http://www.dietzseaweed.com/

http://www.foodculture.dk/Foedevarer/Gastronomi/2015/fremtiden_skal_tages_med_tang.aspx#.VrHe9bIrK70

http://www.dtu.dk/Nyheder/2014/05/Dynamo_Dansk-tang-1-0

APPENDIX

A.1. *In vitro* antioxidant properties of SLE extracts and LLP fraction

The study was performed to determine the effective concetration (EC) of the SLE extracts (WE, WyE, WoE, AE, EE), the LLP fraction (EAF) and phloroglucinol standard (PG) to reach a specific inhibition, i.e. 50%, 30% or absorbance 0.5, according to Table A.1.1.

1. Material methods

1.1. Antioxidant assays

Three assays were applied to determine the EC of the SLE extracts, the LLP fraction and PG: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging, iron chelating and reducing power assay.

Each extract/fraction/PG was dissolved in water in a stock solution of 4 mg/mL. Dilution rows were prepared for the assays. In the DPPH radical scavenging assay concentrations from 1.95 µg/mL to 0.75 mg/mL was evaluated. In the iron chelating assay concentrations from 0.03 mg/mL to 2.50 mg/mL was evaluated. In the reducing power assay concentrations from 3.91 µg/mL to 1.00 mg/mL was evaluated. The antioxidant assay methods were identical to those applied in Paper I and II. 10 to 16 concetrations in triplicates (within the mentioned ranges) were used to conduct the dose/response curves of the extracts/fraction/PG in each assay (Fig. A.1.1-3, means including standard deviations).

1.2. Data treatment

For each triplicate a dose/response curve was prepared and linear regression performed on the linear area of the exponential phase. $1/EC_{50}$, $1/EC_{30}$ and $1/EC_{0.5}$ were determined for DPPH radical scavenging capacity, iron chelating ability and the reducing power, respectively. These values and standard deviations (±SD) were calculated based on these triplicates and stated in Table A.1.1.

For the DPPH radical scavenging assay it was possible to get a response above 50% (Fig. A.1.1), and thereby determine the EC_{50} values. However, in the iron chelating assay WyE, WoE, EAF and PG only reached inhibition levels of maximum 40% (Fig. A.1.2). Therefore EC_{30} was determined in this assay. Whereas the response in the DPPH radical scavenging assay and the iron chelating assay were in percentage (%), the response in the reducing power assay was an absorbance at 700nm (OD_{700}) (Fig. A.1.3). Therefore the $EC_{0.5}$ values were determined in this assay equal to 0.5 in absorbance.

2. Results
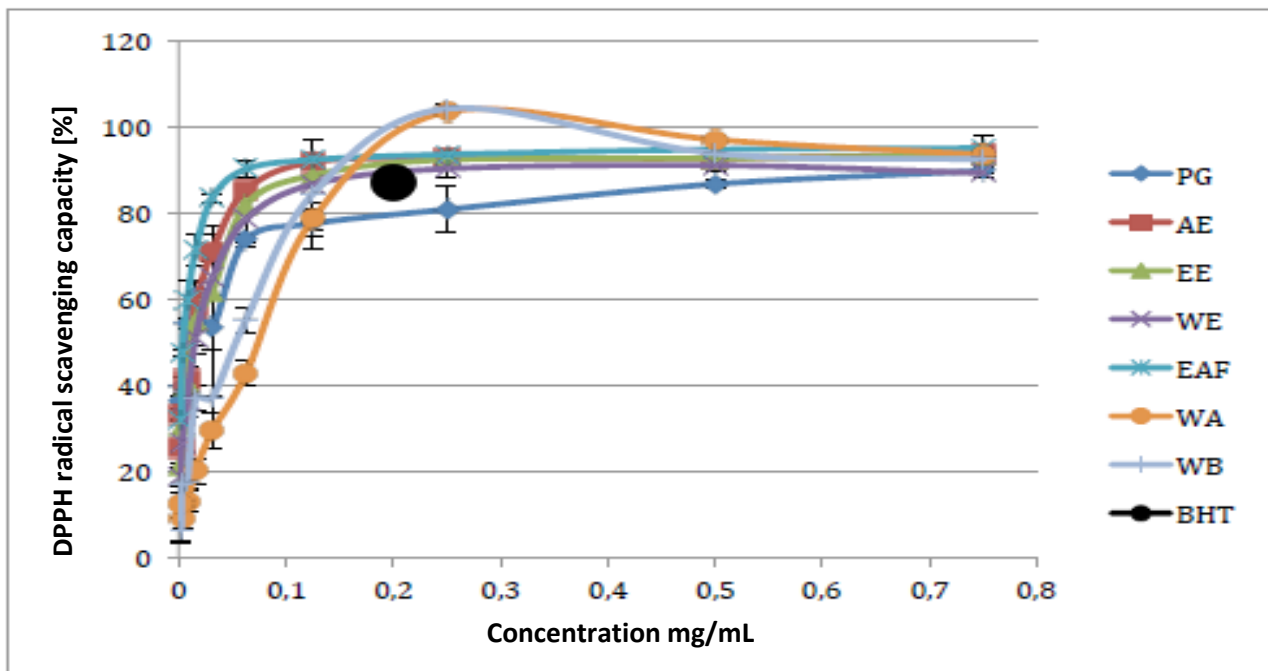
Fig. A.1.1. Dose/response curves of the SLE extracts, LLP fraction and PG in the DPPH radical scavenging assay, including BHT (0.20 mg/mL, assay control). The curves are based on the means of triplicates and the SD (error bars)
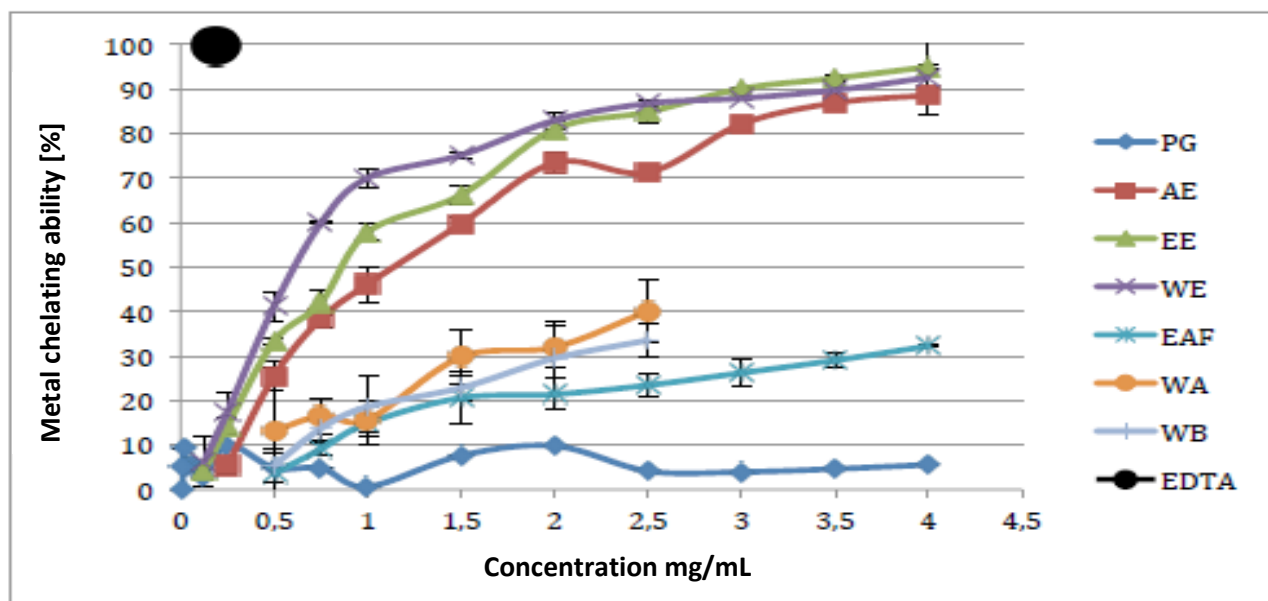
Fig. A.1.2. Dose/response curves of the SLE extracts, LLP fraction and PG in the iron chelating assay, including EDTA (0.19 mg/mL, assay control). The curves are based on the means of triplicates and the SD (error bars)
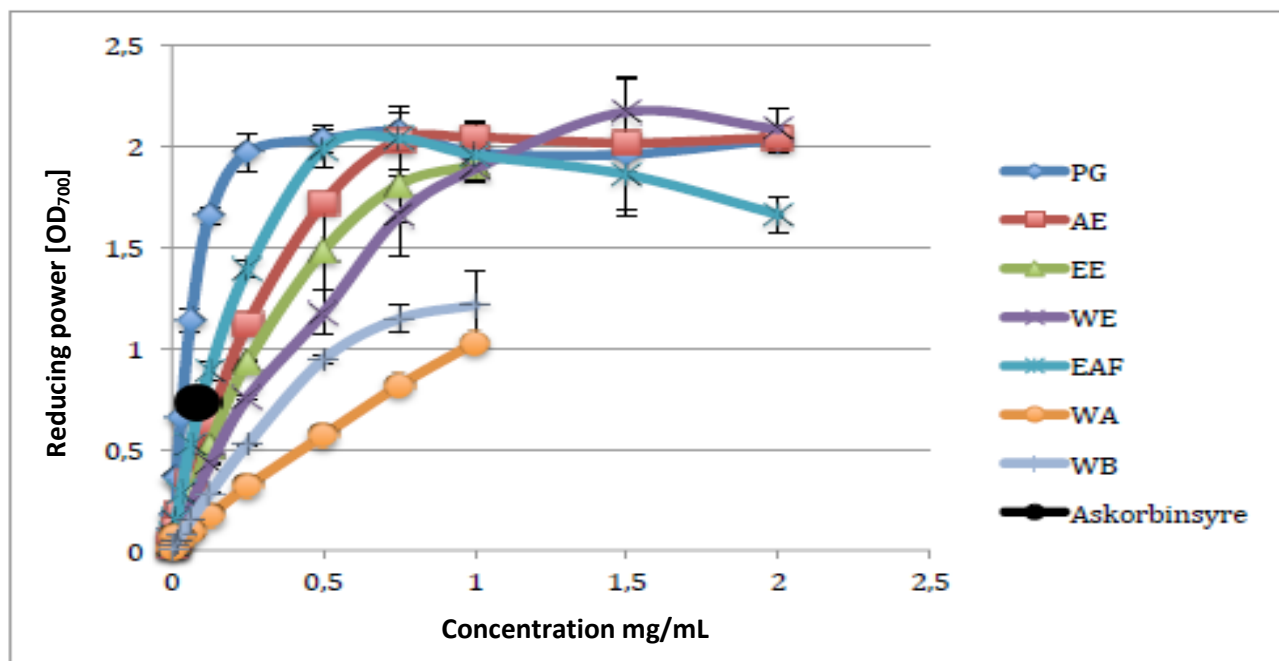
Fig. A.1.3. Dose/response curves of the SLE extracts, LLP fraction and PG in the reducing power assay, including Ascorbic acid (0.09 mg/mL, assay control). The curves are based on the means of triplicates and the SD (error bars)

Table A.1.1. Effective concentrations (EC), mg/mL water, in the three antioxidant assays calculated as $1/EC_{50}$, $1/EC_{30}$ and $1/EC_{0.5}$, respectively, ±SD. n.d. = not detected

SLE extract	LLP fraction	Standard	DPPH radical scavenging capacity $1/EC_{50} \pm SD$	Iron chelating ability $1/EC_{30} \pm SD$	Reducing power $1/EC_{0.5} \pm SD$
WE			53.0±3.6	2.5±0.2	5.1±0.3
WoE			13.8±1.0	0.6±0.1	2.2±0.0
WyE			16.9±2.2	0.5±0.1	4.0±0.1
AE			68.2±0.3	1.5±0.1	8.4±0.2
EE			75.3±15.4	2.1±0.3	7.9±0.0
	EAF		169.3±34.8	0.3±0.0	13.5±0.4
		PG	120.2±10.8	n.d.	37.5±0.8

APPENDIX

A.2. Evauation of pressurized liquid extraction for extraction of phlorotannins from Danish *F. vesiculosus*

The aim of this study was to evaluate the possibility of using pressurised liquid extraction for extraction of phenolic compounds as an alternative "green" method to the traditional solvent extraction. The methods and results are stated in this appendix.

1. Material and method

1.1. Algal material

Fucus vesiculosus specimens were collected at the sandy beaches of Hou havn (55°54'39''N 10°14'59''E) (September 2009). Fresh seaweeds were washed with distilled water and snails and epiphytes were removed. Immediately hereafter the rinsed seaweeds were placed in a freezer (-40 °C) until further use. The dry weight (dw) of the clean seaweed was determined by drying approximately 2 g seaweed in an oven at 70°C for 24h. This was carried out in triplicates. The dw was 37.7±1.2%.

1.2. Pressurised liquid extraction (PLE)

Two PLE extracts were conducted, PLEW using 100% water and PLEE using 70% ethanol. Prior to extraction, the clean *F. vesiculosus* plants was quickly frozen using fluent nitrogen and grinded by blending (Kitchen blender, 800W). 1.5 g of grinded seaweed was weighed into 11 mL extraction cells, packed with filter Extractions were performed using a Dionex ASE 200 system (Thermo Fisher, Germering, Germany). Water and ethanol used for extraction, were sonicated for 40 min prior to extraction to remove oxygen. Extractions were performed at 125°C for 3 min at a pressure of 1500 psi. Prior to each extraction the extraction cell was heated for 7 minutes. The extractions were performed in triplicates (PLEW1-3 and PLEE1-3). The schematic overview of the extraction process is shown in Fig. A.2.1.

The extraction was performed as follows: The extraction cell was loaded into the oven; the cell was filled with liquid under pressure; heating time was applied; a static extraction with all system valves closed was performed; the cell was cleaned (30% of cell volume using extraction solvent); the cleaning solvent was removed from the extraction cell using N_2 and the system was depressurized. Between extractions, cleaning of the complete system was carried out to prevent any carry-over of extracted compounds. The PLE extracts were in liquid form and approximately 25 mL was obtained by extraction. The PLE extracts were protected from light and stored at -80°C until analysis.
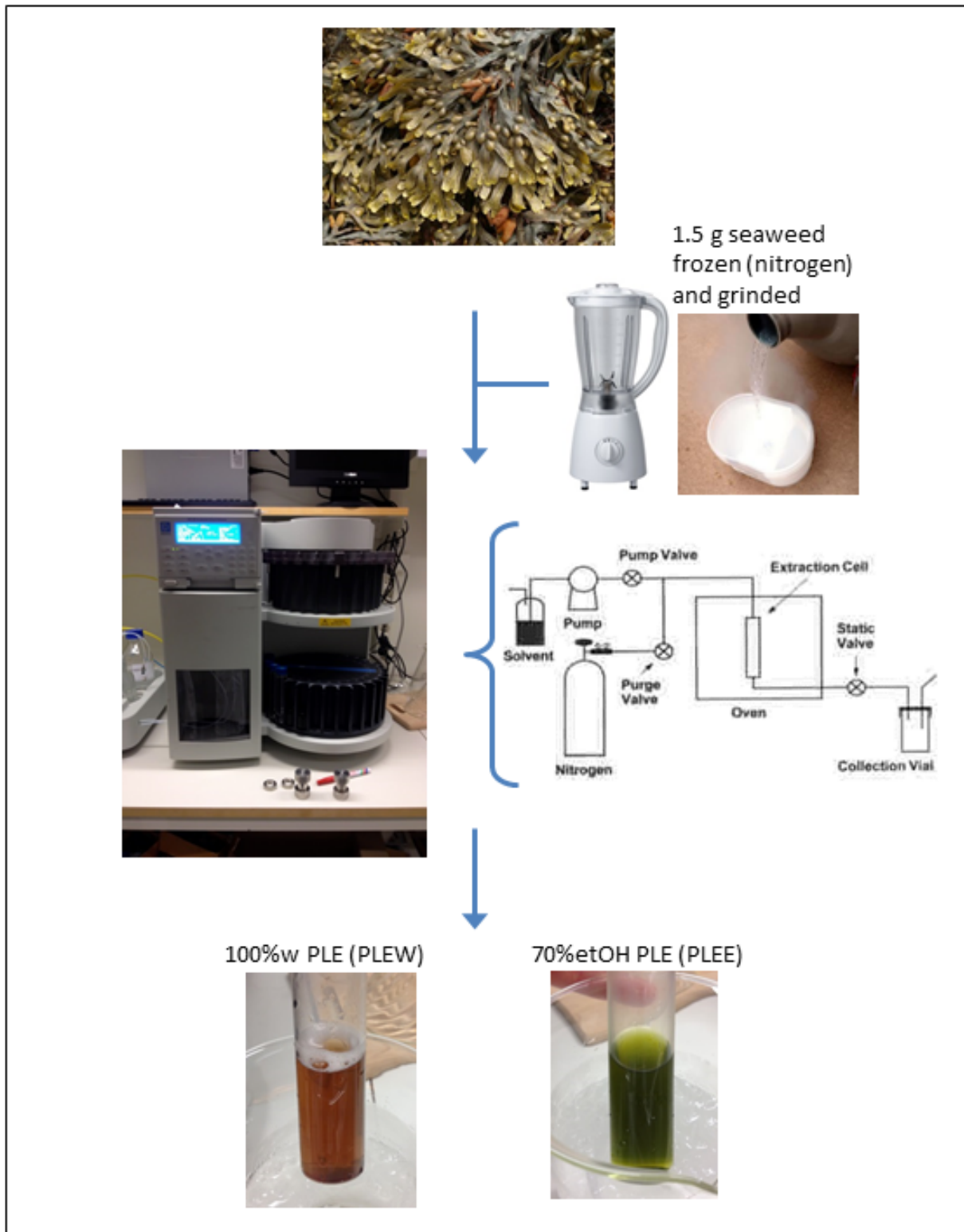
Fig. A.2.1. Schematic overview of the pressurized liquid extraction (PLE) process performed to obtain PLEW and PLEE

1.3. Total phenolic content

The total phenolic content (TPC) of the conducted PLE extracts were determined using a Folin-Ciocaltau assay, identical to the one applied in Paper I and II. Undiluted PLE extracts were used to determining TPC. 20 µL PLE extract were added to the microtiter plate together with 150 µL reagent and natrium bicarbonat. TPC was calculated from the standard curve using gallic acid. The results are stated as g GAE/100 mL PLE extract.

1.4. Antioxidant assays

DPPH radical scavenging, iron chelation and reducing power assay were applied to determine the antioxidant properties of the PLE extracts. The antioxidant assay methods were identical to the ones applied in Paper I and II. Three concentrations were used (depending on assay) see Table A.2.1. The extracts were weighed and diluted in water to obtain these concentrations (mg PLE extract/mL water).

1.5. Data treatment

Means were calculated for both TPC and antioxidant assays. TPC was based on the extraction triplicates, therefore the SD is big. The antioxidant properties were only determined for one of the triplicates (PLEW1 or PLEE1) (gives small SD). Standard deviations (±SD) were given. All results are stated in Table A.2.1.

2. Results

Table A.2.1.TPC (g GAE/100 mL extract) and antioxidant properties determined at different concentrations of PLEW and PLEE.

PLE extract	TPC	Antioxidant properties		
	g GAE/100 mL PLE extract	DPPH radical scavenging capacity*, %	Iron chelating ability**, %	Reducing power**, OD_{700}
PLEW	2.4±1.3	49.2±1.2	18.8±7.2	0.2±0.0
		77.5±0.4	21.4±5.1	0.2±0.0
		83.7±3.1	17.8±4.7	0.3±0.0
PLEE	2.1±0.5	40.4±1.1	9.1±4.1	0.1±0.0
		71.7±1.2	7.5±0.4	0.3±0.2
		77.3±1.2	6.0±4.9	0.2±0.0

*Concentration 0.2, 0.6 and 1.0 mg/mL. **Concentration 1.0, 1.5 and 2.0 mg/mL*

PAPER I

Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts *in vitro* and in fish-oil-enriched milk and mayonnaise

Hermund, DB, Yeşiltaş, B, Honold, P, Jónsdóttir, R, Kristinsson, HG, & Jacobsen, C

Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts *in vitro* and in fish-oil-enriched milk and mayonnaise

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ARTICLE INFO

ABSTRACT

Bioactive compounds from *Fucus vesiculosus* were extracted and their antioxidant efficacy in fish-oil-enriched foods was evaluated. Water extract (WE) and ethyl acetate fraction (EAF) were obtained and characterised. Furthermore, WE and EAF were added to fish-oil-enriched milk or mayonnaise. Oxidation during storage was evaluated. EAF showed highest phenolic and pigment contents compared with WE. Antioxidant efficacy was found to be dependent on the concentration in the foods. Furthermore, high antioxidant activity in the foods was related to high radical scavenging, high or moderate metal chelating ability, as well as high phenolic and carotenoid contents.

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1. Introduction

The demand for functional foods enriched with n-3 LC PUFA of marine origin is increasing due to the health benefits of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Covert, 2009; Horrocks & Yeo, 1999). However, enrichment with n-3 LC PUFA rich fish oil decreases the oxidative stability of such foods, leading to the development of undesirable off-flavours and reduced shelf life (Jacobsen et al., 2000; Let, Jacobsen, Frankel, & Meyer, 2003; Let, Jacobsen, & Meyer, 2007). Therefore, it is necessary to prevent these quality changes and sustain consumer acceptance by the addition of antioxidants.

Natural antioxidants can be derived from various plants and marine algae. Many natural antioxidants show great potential for improving oxidative stability of food products. Furthermore, these compounds also have a broad array of additional health-promoting benefits (Hata, Nakajima, Uchida,

Hidaka, & Nakano, 2001; Kim et al., 2009). Seaweed contains several bioactive compounds, such as pigments (carotenoids, chlorophylls and tocopherols), sulphated polysaccharides (fucoidan), amino acids, and mono- and polyphenols (Farvin & Jacobsen, 2013; Holdt & Kraan, 2011; Kim, Kim, Nam, & Kong, 2012; Rodríguez-Bernaldo de Quirós, Frecha-Ferreiro, Vidal-Pérez, & López-Hernández, 2010; Rodriguez-Jasso, Mussatto, Pastrana, Aguilar, & Teixeira, 2014). Brown algae *Fucus vesiculosus* produces high amounts of polyphenolic secondary metabolites, phlorotannins (Chkhikvishvili & Ramazanov, 2000; Wang et al., 2012; Wang, Jónsdóttir, & Ólafsdóttir, 2009), which are the main contributors to the overall antioxidant activity of extracts from brown algae (Chkhikvishvili & Ramazanov, 2000; Farvin & Jacobsen, 2013; Jiménez-Escrig, Jiménez-Jiménez, Pulido, & Saura-Calixto, 2001; O'Sullivan et al., 2011; Wang et al., 2009, 2012). Phlorotannins consist of up to eight interconnected rings (phloroglucinol units). This structure makes phlorotannins more antioxidative than monophenols and polyphenols with less phenolic rings and therefore also fewer functional —OH groups (Wang et al., 2009).

The antioxidant properties of *F. vesiculosus* have been widely studied in simple *in vitro* assays (Farvin & Jacobsen, 2013; Wang et al., 2009, 2012). In contrast, studies on the antioxidant activity of *F. vesiculosus* extracts to protect against oxidation are limited (Halldorsdottir, Sveinsdottir, Gudmundsdottir, Thorkelsson, & Kristinsson, 2014).

Mayonnaise and milk are both oil-in-water (o/w) emulsions in which lipid oxidation typically is initiated at the interface between oil and water and progresses in the oil phase. Fortification with fish oil increases the oxidation rate since the fat phase becomes more susceptible to oxidation (Frankel, 1998). Typical lipid oxidation initiators in foods are trace metals. Trace metals such as iron and copper can react with unsaturated lipids to form lipid radicals (alkyl radicals) or lead to degradation of peroxides to alkoxyl radicals. In foods, trace metals are naturally present in the water phase. A large amount of iron in mayonnaise stems from egg yolk, which is traditionally used as the emulsifying agent in this type of product. In native egg yolk, the major proportion of iron is bound to the protein phosvitin (Causeret, Matringe, & Lorient, 1991). However, when the egg yolk is incorporated into mayonnaise, the low pH (pH 4) is responsible for breaking the ion bridges between iron and phosvitin, which releases iron, and enables it to participate in lipid oxidation (Jacobsen, Schwarz, Stöckmann, Meyer, & Adler-Nissen, 1999).

Several studies on antioxidant addition in emulsion systems with low pH (pH 3) and in fish-oil-enriched mayonnaise have been carried out (Alemán et al., 2015; Haahr & Jacobsen, 2008; Jacobsen et al., 2001). Moreover, fish-oil-enriched milk emulsions have also been studied (Alemán et al., 2015; Sørensen, Lyneborg, Villeneuve, & Jacobsen, 2014). It has been observed that the efficacy of antioxidants not only depends on the antioxidative properties but also on the location of the antioxidants in the o/w emulsion. This is due to the fact that antioxidants located at the oil–water interface are more effective than antioxidants located in the oil droplets or in the continuous water phase. Nevertheless, antioxidants should have good free radical scavenging properties. Scavenging of pre-existing free radicals as well as those which are formed during storage of food products is crucial to limit lipid oxidation.

Furthermore, the synthetic chelator ethylenediaminetetraacetic acid (EDTA) has been found to be the most efficient inhibitor of metal-catalysed oxidation in fish-oil-enriched and mayonnaise (Jacobsen et al., 2001; Let et al., 2003). This is due to its ability to prevent metal-catalysed degradation of peroxides by metal chelation (Haahr & Jacobsen, 2008).

The aim of this study was to evaluate the antioxidant properties of bioactive compounds extracted from Icelandic brown algae *F. vesiculosus* and to relate these properties to their efficacy to inhibit lipid oxidation in both fish-oil-enriched mayonnaise and milk.

2. Materials and methods

Pasteurised milk (0.5% and 1.5% fat), salt and sugar were purchased from the local supermarket. Whole egg powder was purchased from Sanovo foods (Odense, Denmark). Yellow colour (E160a), lemon juice concentrate and Stabilizer Xanthan gum FG (E415) were obtained from Crown Food (Mørkøv, Denmark). Potassium sorbate (E202) was purchased from Merck (Darmstadt, Germany). Rapeseed oil and fish oil (cod liver) were supplied by Maritex A/S (TINE, BA, Sortland, Norway). The fish oil, used in the milk preparation, had a PV of 0.24 meq peroxides/kg oil and a tocopherol content of 237 µg α-tocopherol/g oil, 99 µg γ-tocopherol/g oil and 44 µg δ-tocopherol/g oil. The fatty acid composition was as follows: 14:0, 3.2%; 16:0, 9.2%; 16:1 n-7, 8.6%; 16:3 n-4, 0.5; 18:0, 2.0%; 18:1 n-9, 16.7%; 18:1 n-7, 4.3%; 18:2 n-6, 1.8%; 18:3 n-3, 0.9%; 18:4 n-3, 2.6%; 20:1 n-11, 12.8%; 20:4 n-3, 0.7%; 20:5 n-3 (EPA), 9.3%; 22:1 n-11 + n-9, 6.7%; 22:5 n-3, 1.1% and 22:6 n-3 (DHA) 11.6%. The fish oil, used in the mayonnaise preparation, had a PV of 0.16 meq peroxides/kg oil and a tocopherol content of 234 µg α-tocopherol/g oil, 3.7 µg β-tocopherol/g oil, 109 µg γ-tocopherol/g oil and 45 µg δ-tocopherol/g oil. The fatty acid composition was as follows: 14:0, 3.5%; 16:0, 10.0%; 16:1 (n-7), 9.2%; 16:3 (n-4), 0.5%; 18:0, 2.0%; 18:1 (n-9), 16.9%; 18:1 (n-7), 4.8%; 18:2 (n-6), 1.9%, 18:3 (n-4), 0.9%; 18:4 (n-3), 2.7%; 20:1 (n-11 + n-9), 12.43%; 20:4 (n-3), 0.7%; 20:5 (n-3) (EPA), 9.2%; 22:1 (n-11), 0.8%; 22:1 (n-9), 5.3%, 22:5 (n-3), 1.0% and 22:6 (n-3) (DHA) 10.3%. The rapeseed oil, used in the mayonnaise preparation, had a PV of 0.24 meq peroxides/kg oil and a tocopherol content of 163 µg α-tocopherol/g oil and 253 µg γ-tocopherol/g oil. The fatty acid composition was as follows: 16:0, 4.5%; 18:0, 1.65%; 18:1 (n-9), 57.0%; 18:1 (n-7), 2.2%; 18:2 (n-6), 19.9%; 18:3 (n-3), 10.1%; 20:0, 0.6%; 20:1 (n-11) + (n-9), 1.4%.

All solvents used were of high-performance liquid chromatography (HPLC) grade and purchased from Lab-Scan (Dublin, Ireland). Lactic acid (60%), acetic acid (20%) and the external standards were purchased from Sigma Aldrich (Steinheim, Germany).

2.1. *Extraction and fractionation*

The extraction and fractionation was performed by Matís in Iceland according to Wang et al. (2009; 2010; 2012). The seaweed was collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in 2011. At the collecting spot the seaweed was washed with clean seawater to

remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water and wiped with paper towel. The samples were freeze-dried, pulverised into powder and stored at −80 °C prior to extraction.

The water extract (WE) was produced as follows: Five grams of the algal powder was mixed with 100 mL of distilled water (v/v), incubated in a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, USA) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2168 *g* for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. The ethyl acetate fraction (EAF) was produced as follows: Two grams of dried algal powder were dispersed in 200 mL 80% ethanol (v/v) and incubated in the platform shaker for 24 h at 200 rpm and at room temperature. The extract was concentrated in vacuum to a small volume, and the residue was suspended in a mixture of methanol and water (40:30, v/v) and partitioned three times with n-hexane, ethyl acetate, and 1-butanol successively. After the solvent was removed and freeze dried, four fractions were obtained, the n-hexane, ethyl acetate, and 1-butanol soluble fractions and an aqueous residue. Only the EAF was used in this study because of its high phenolic content.

Each extraction was conducted in duplicate. WE and EAF were stored at −80 °C until used for application and analysis.

2.2.   Trace metals

WE and EAF were destructed prior to metal analysis: 0.5 g extract/fraction was mixed with 5 mL HNO_3 (68%), 3 mL H_2O_2 (30%) and 0.5 mL HCl (37%). Two reference samples with fish protein certified reference material for trace metals (DORM-3) (National Research Council, Ottawa, ON, Canada) fish protein were prepared the same way. Destruction was carried out in a destruction oven (Multiwave 3000, Anton Paar, Graz, Austria), in which the samples were heated for 10 min at 1400 W. After destruction the samples were kept at room temperature for 20 min before being transferred to 10 mL volumetric flasks and filled with water. Prior to analysis, samples were diluted to reach acidity below 2% HNO_3 and 1% HCl. Analysis was performed using Inductively Coupled Plasma Mass Spectrometry (ICPMS) (ICPMS model 7500ce, Agilent Technologies, Wilmington, DE, USA) equipped with a Scott-type spray-chamber and a concentric nebuliser with a CETAC autosampler (ASX-500, CETAC Technologies, Omaha, NE, USA). The analysis was done on the isotopes 57Fe and 63Cu with the ICPMS in collision-reaction-cell (CRC) mode using helium as cell-gas for interference reduction. The calibration was done using external calibration with standard solutions in the concentration range from 0 to 200 µg/L for both elements. The calibration standards were prepared by dilution of certified single-element stock solutions (SCP Science, Courtaboeuf, France) of 1000 mg/L. Experiment was performed in duplicates.

2.3.   Protein

The %protein (w/w) was determined by Kjeldahl method (AOCS, 1995). Distillation was carried out on 0.3 g WE or EAF, two Kjeltabs CT (Thompson & Capper, Ltd, England) and 20 mL H_2SO_4 per tube, using 2100 Kjeltec distillation unit (Foss, Hillerød,

Denmark). Samples were heated for 1 h up to 440 °C and kept at 440 °C for 30 min. After distillation, the distillates were titrated on Dosimat 665 (Metrohm, Herisau, Switzerland) with 0.1M NaOH until indicator (Andersen indicator) turned green. A conversion factor of 5.38 was used for converting nitrogen to protein in brown algae (Lourenço, Barbarino, De-Paula, da S. Pereira, & Marquez, 2002). The analysis was performed in duplicate and two blank samples without WE or EAF were included.

2.4.   Pigments

Before WE or EAF were analysed the pigments were extracted. The extraction was performed as follows: 10 mg extract/fraction, 1 mL acetone and 1 µL of internal standard (20 ppm tocopherol acetate) were mixed. The solution was sonicated for 1 h on ice in the dark. After sonication samples were centrifuged for 10 min at 13,684 *g*. The supernatants were removed and stored and the extraction method was repeated one time. The supernatants were pooled and filtered with a hydrophobic filter (pore size 0.22 µm).

The analysis of pigments was based on an HPLC method of Van Heukelem and Thomas (2001) with some modifications. The pigment extracts were transferred to HPLC-vials and the pigments were separated with a gradient consisting of; A: 70% methanol and 30% buffer (0.028 M tetrabutylammonium acetate in water, pH = 6.5) and B: methanol. The mobile phase starting at 5% B increasing to 90% in 27 min and held for 7 min at 90%, 90 to 100% in 1 min where it was held for 3 min, decreasing from 100% to 90% in 2 min, returned to 5% in 6 min and held there for 2 min. Injection volume was 100 µL and the flow rate was 1.1 mL/min at 60 °C. The pigments were quantified as equivalent of astaxanthin (carotene) and chlorophyll a (Chlorophylls). Pigments were detected by fluorescence using 450 and 440 nm as detection wavelengths. For the internal standard the wavelength was 222 nm. Experiment was performed in duplicate and results were expressed in µg pigments/mg dry WE or EAF.

2.5.   Total phenolic content (Folin–Ciocalteu)

The total phenolic content (TPC) of WE and EAF was quantified according to the method of Farvin and Jacobsen (2013) with modifications. One hundred microlitres of sample solution (dissolved in methanol, concentration range from 0.1 to 8.0 mg/mL) were mixed with 0.75 mL of Folin–Ciocalteu reagent (10% in distilled water). After 5 min, 0.75 mL of sodium carbonate (7.5% in distilled water) was added. The samples were incubated for 1.5 h at room temperature in the dark. To a microtitreplate, 200 µL sample were transferred and measured at 725 nm using a UV–vis spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). A standard curve with serial gallic acid solutions (0–100 µg/mL) was used for calibration. The results are expressed as grams gallic acid equivalents (GAE)/100 g WE or EAF in methanol.

2.6.   Antioxidant properties

For determination of the antioxidant properties of WE or EAF, three antioxidant assays; DPPH scavenging activity, Fe^{2+}

chelating activity and reducing power were used. WE, EAF or control were prepared in four different concentrations (1.0, 1.5 and 2.0 mg/mL methanol).

2.6.1. DPPH scavenging activity

The ability of WE or EAF to scavenge radicals and H-atom donation was determined according to the method described by Yang, Guo, and Yuan (2008) modified using multiplate reader. One hundred fifty microlitres extract/fraction solution were mixed with 150 μL 0.1 mM DPPH (in 96% ethanol). Two hundred microlitres of the mixture were added to a microtitre plate and the absorbance was measured at 20 °C at 517 nm after 30 min (A_s). Triplicate measurements were performed and butylated hydroxytoluene (BHT) was included as a positive control. A sample blank was made with DPPH but without extract/fraction solution (A_b) and a sample control was made without DPPH but with extract/fraction solution (A_0). The results were given as per cent inhibition:

$$Inhibition\,[\%] = \left(1 - \frac{A_s - A_0}{A_b}\right) \cdot 100.$$

2.6.2. Fe²⁺ chelating activity

The Fe^{2+} chelating activity was determined according to the method described by Farvin, Baron, Nielsen, and Jacobsen (2010) with modifications. One hundred microlitres of the extract/fraction solution and 135 μL distilled water were added to the microtitre plate. Five microlitres of 2 mM ferrous chloride were mixed into the solution to initiate the reaction. After 3 min 10 μL 5 mM ferrozine were mixed into the solution and the absorbance was read at 562 nm (A_s). Triplicate measurements were performed and ethylenediaminetetraacetic acid (EDTA) was included as a positive control. A sample blank was made without extract/fraction solution (only iron and ferrozine) (A_b) and a sample control was made with only the extract/fraction solution (A_0). The Fe^{2+} chelating activity in per cent was calculated as follows:

$$Fe^{2+}\mathbf{chelating\ activity} = \left(\frac{A_b - (A_s - A_0)}{A_b}\right) \cdot 100.$$

2.6.3. Reducing power

The reducing power was determined according to the method described by Yang et al. (2008) with modifications. Two hundred microlitres of the extract/fraction solution were mixed with 200 μL 0.2 M phosphate buffer (pH 6.6) and 200 μL 1% potassium ferricyanide and incubated in a water bath at 50 °C for 20 min. Two hundred microlitres of the incubated solution were then mixed with 10% TCA. Two hundred twenty eight microlitres of this solution was diluted with 228 μL distilled water, 46 μL 0.1% ferric chloride were added. After 10 min of incubation at 20 °C 200 μL mixture were added to the microtitreplate and the absorbance was measured at 700 nm (A_s). Triplicate measurements were performed and ascorbic acid (AscA) was included as a positive control. A sample control was made with only the extract/fraction solution (A_0). The results were given in OD_{700} ($A_s - A_0$).

2.7. Production of fish-oil-enriched milk

Conventional homogenised milks with fat content of 0.5 and 1.5% were mixed (1:1v/v) to obtain a total milk fat content of 1%. The production of the milk was made based on research results from Let, Jacobsen, Sørensen, and Meyer (2007). Subsequently, the milk was heated to 72 °C for 15 s. Hereafter, fish oil (0.5% w/w) and the dry WE or EAF in concentrations of 1.0 (1), 1.5 (2) and 2.0 (3) g/kg milk were added to the heated milk. The mixture was immediately hereafter homogenised using a two valve table homogeniser from GEA Niro Soavi Spa (Parma, Italy), with the pressure set at 250 bar. The mixture was recirculated four times to achieve a stable emulsion (Sørensen et al., 2007, 2012). Milk without the addition of WE or EAF was also made using the same procedure (Control). The milk emulsions were stored in the dark in five 250 mL sterilised bottles, one for each storage time, at 5 °C. Samples were taken at day 0, 3, 6, 9 and 12 and subdivided into 50 mL brown bottles, flushed with N_2 and stored at –40 °C until analyses (up to 12 days). An exception was sample droplet size measurements, which was performed on day 1 and 12 without prior freezing.

2.8. Production of fish-oil-enriched mayonnaise

The mayonnaise batches of 1000 g were prepared under vacuum using a Stephan Universal mixer (Stephan UMC5, Hameln, Germany). The production of mayonnaises under these conditions assures physically stable emulsions as has been proven in previous studies (Meyer & Jacobsen, 1996). Each batch contained by weight 61.48% rapeseed oil, 15.37% fish oil, 18.25% water, 2.00% heat stable egg yolk powder, 0.09% lemon juice, 1.30% sugar, 0.60% salt, 0.005% Yellow colour (E160a), 0.08% lactic acid (80%), 0.72% acetic acid, 0.05% Xanthan gum FG and 0.08% potassium sorbate adding up to approximately 100%. Egg yolk powder, potassium sorbate, yellow colour, salt, sugar and dry WE or EAF in concentrations of 1.0 (1), 1.5 (2) and 2.0 (3) g/kg were mixed into the water. The water mixture was blended on Stephan mixer for 15 s under vacuum. The two oils (rapeseed and fish oil) were mixed together and a small portion was taken to dissolve the Xanthan gum in before this was added to the water mixture. The mixture was blended on Stephan mixer for another 15 s under vacuum. To the remaining oil, the lemon juice, lactic acid and acetic acid were added. The oil mixture was then slowly poured (2 min) into the Stephan mixer without vacuum, to form an emulsion. Finally, the emulsion was mixed under vacuum for 30 s. Mayonnaise without the addition of WE or EAF was also made in the same procedure (Control). Mayonnaise with sample code EAF3 did not become an emulsion and was not analysed.

Mayonnaises were stored in 300 mL brown bottles, at 20 °C for 28 days in the dark. Samples were taken at 5 different time points (day 0, 7, 14, 21 and 28) and subdivided into 150 mL brown bottles, flushed with N_2 and stored at –40 °C until analysis (up to 28 days). An exception was sample droplet size measurements, which were performed on day 7 and 28 without prior freezing.

2.9. Droplet size distribution

The size of fat globules in the two o/w emulsion systems was determined by laser diffraction using a Mastersizer2000 (Malvern Ins., Worcestershire, UK).

Droplets of milk emulsions were added directly to recirculating water (2800 rpm) reaching an obscuration of 14–16%. The

set-up used was the Fraunhofer method, which assumed that all sizes of particles scatter light with the same efficiency and that the particles are opaque and transmits no light. Measurements were performed on day 1 and 12.

The mayonnaise was diluted 1:9 in SDS buffer (10 mM NaH$_2$PO$_4$, 5 mM SDS, pH 7) and sonicated (2 × 20 min at room temperature) prior to analysis. Droplets of the diluted mayonnaise was added to recirculating water (3000 rpm) reaching an obscuration of 12–14%. The refractive index (RI) of sunflower at 1.469 and water at 1.330 were used as particle and dispersant, respectively. Measurements were performed on day 7 and 28.

The measurements were performed in triplicates. Results are given as surface area mean diameter $D[3,2] = \sum d^3/d^2$ (Rawle, 1996).

2.10. Extraction of lipids from WE or EAF and milk, and separation of oil phase in mayonnaise

Prior to analysis of peroxide value, the fatty acid composition and tocopherol content the oil phase was extracted from the milk, WE or EAF, or separated from the mayonnaise. Lipids were extracted from fish oil enriched milk, WE or EAF according to the method described by Iverson, Lang, and Cooper (2001) based on the method of Bligh and Dyer (1959). There were some modifications for extraction of lipids from the WE or EAF where 50 mg of each were used for extraction. Lipid extracts from WE or EAF were only used for analysis of tocopherol. For each sample two oil extractions were performed.

Separation of oil from mayonnaise was done by freezing mayonnaise for a minimum of 24 h at –80 °C. The mayonnaise was defrosted and centrifuged (1620 g, 10 min, 4 °C). The oil phase was then collected and used for the different analyses.

2.11. Fatty acid composition (fatty acid methyl esters, FAME)

The fatty acid composition of the oil phases was determined by FAME method and analysed by GC-FID. The oil phase from mayonnaise and the Bligh and Dyer (1959) lipid extract from milk were weighted in vials. Toluene and heptane with intern standard (C23:0) (400 μL, 1:3v/v) were added and the lipids were methylated in a one-step procedure using a microwave oven (Multiwave3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave were 5 min at 500 Watt followed by 10 min cooling. The FAMEs were dissolved in heptane and analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to AOCS (1998). For separation DB127-7012 column (10 m × ID 0.1 mm × 0.1 μm film thickness, Agilent Technologies) was used. Injection volume was 0.2 μL in split mode (1:70). The initial temperature of the GC-oven was 160 °C. The temperature was set to increase gradually being as follows: 160–200 °C (10.6 °C/min), 200 °C for 0.3 min, 200–220 °C (10.6 °C/min), 220 °C for 1 min, 220–240 °C (10.6 °C/min) and 240 °C for 3.8 min. The determination was made in duplicates. Results were given in % and a Relative Decrease in Area% (RDA%) of EPA and DHA was calculated. The measurements were performed at storage day 0 and 12 for milk and on storage day 0 and 28 for mayonnaise.

2.12. Primary oxidation products: peroxide value (PV)

PVs of the lipid extract of milk or oil phase from mayonnaise were determined. This was done according to the method by Shantha and Decker (1994), based on the formation of an iron–thiocyanate complex. The coloured complex was measured spectrophotometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA). The analyses were done in duplicates and the results are expressed in milliequivalents peroxides per kg oil (meq O$_2$/kg).

2.13. Tocopherol content

The oil phase from mayonnaise and lipid extract from either milk, WE or EAF were (after evaporation under nitrogen) dissolved in heptane and analysed by HPLC (Agilent 1100 Series, Agilent Technology) according to AOCS (1998) to quantify the contents of α-, β-, γ- and δ-tocopherols in the samples. These tocopherol homologues were separated using a silica column (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 μm silica film). A stock solution added 10 mg tocopherols (mixture of α-, β-, γ- and δ-tocopherols) per litre was prepared and used for quantification. The analyses were done in duplicates and results were reported as μg tocopherol/g milk or oil.

2.14. Analysis of secondary oxidation products, volatiles

Tenax GR™ packed tubes were used to collect volatile compounds by dynamic headspace. The collection of the volatile compounds was carried out using 4 g of mayonnaise sample (incl. 30 mg int.std., 4-methyl-1-pentanol) and 8 g of milk sample (incl. 30 mg int.std. and antifoaming agent). The volatile secondary oxidation products were collected at 60 °C for mayonnaise and 45 °C for milk followed by flushing with nitrogen (flow of 150 mL/min) for 30 min to remove water from the Tenax tube. For the mayonnaise sample, volatile acids were removed by 0.1 g KOH during the headspace collection, by connection of an S-tube with KOH, as described by Hartvigsen, Lund, Hansen, and Hølmer (2000). The trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin-Elmer, Norwalk, CT) connected to an Agilent 5890 IIA model gas chromatograph equipped with a HP 5972 mass selective detector. Chromatographic separation of volatile compounds was performed on a DB1701 column (30 m × ID 0.25 mm × 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min). The temperature programme for mayonnaise was as follows: 3 min at 35 °C, 3 °C/min from 35 to 120 °C, 7 °C/min to 120–160 °C, 15 °C/min 160–200 °C and hold for 4 min at 200 °C. For milk the temperature programme was: 5 min at 45 °C, 1.5 °C/min from 45 to 55 °C, 2.5 °C/min from 55 to 90 °C, 12 °C/min from 90 to 200 °C and hold for 4 min at 200 °C.

The auto sampler collectors setting details are: 9.2 psi, outlet split: 5.0 mL/min, desorption flow: 60 mL/min. Both for mayonnaise and milk the analysis was performed in triplicate and the results are given in ng/g of mayonnaise or milk.

The quantification of the different volatiles was done by the use of a calibration curve prepared from external standards. Solutions with external standards at different concentrations were prepared and added to fresh milk or mayonnaise

Table 1 – Characterisation of WE and EAF. Total phenolic content, the *in vitro* antioxidant properties measured at three concentrations (1, 1.5 and 2 mg dry WE or EAF/mL methanol), tocopherol content, pigments and trace metals.

			WE	EAF	Control[e]
Total phenolic content		[mg GAE/ 100g dry WE or EAF]	18.4 ± 0.1	26.5 ± 1.2	–
Antioxidant properties	*Radical scavenging activity [%]*	1.0 mg/mL	93.5 ± 0.7 [ax]	97.3 ± 0.1 [bx]	87.1 ± 0.4 [cx]
		1.5 mg/mL	93.6 ± 0.5 [ax]	97.1 ± 0.8 [bx]	86.2 ± 0.1 [cx]
		2.0 mg/mL	92.1 ± 0.1 [ay]	93.6 ± 1.2 [by]	87.3 ± 0.8 [cx]
	Metal chelating ability [%]	1.0 mg/mL	94.8 ± 6.1 [ax]	30.0 ± 3.2 [bx]	50.8 ± 17.7 [cx]
		1.5 mg/mL	75.6 ± 10.8[ax]	34.3 ± 7.2 [bx]	64.4 ± 9.3 [acy]
		2.0 mg/mL	84.7 ± 6.6 [ax]	32.7 ± 10.4[bx]	93.6 ± 0.4 [acz]
	Reducing power [OD$_{700}$]	1.0 mg/mL	1.0 ± 0.2 [ax]	1.0 ± 0.0 [ax]	1.0 ± 0.2 [ax]
		1.5 mg/mL	0.8 ± 1.1 [ax]	1.1 ± 0.1 [bx]	1.5 ± 0.2 [cy]
		2.0 mg/mL	1.0 ± 0.1 [ax]	1.1 ± 0.1 [ax]	1.0 ± 0.1 [axz]
Tocopherol content	*Alpha*	[µg toc/g dry WE or EAF]	19.0 ± 1.9	8.3 ± 0.1	–
	Beta		2.9 ± 0.0	3.5 ± 0.0	–
	Gamma		6.2 ± 0.2	5.1 ± 0.6	–
	Delta		24.5 ± 1.2	43.4 ± 2.0	–
Pigments	*Chlorophyll C2*	[mg/mg dry WE or EAF]	0.5 ± 0.0	15.8 ± 2.6	–
	Chlorophyllide a		nd[d]	10.0 ± 1.5	–
	19-But-Fucoxanthin		nd[d]	43.0 ± 1.9	–
	Fucoxanthin		nd[d]	9.4 ± 3.7	–
	Prasinoxanthin		1.26 ± 0.01	4.0 ± 0.4	–
	Diadinoxanthin		0.22 ± 0.04	6.4 ± 0.1	–
	Dinoxanthin		0.20 ± 0.05	4.7 ± 0.4	–
	Zeaxanthin		0.2 ± 0.1	nd[d]	–
	Lutein		nd[d]	0.7 ± 0.0	–
	Dihydrolutein		0.3 ± 0.0	nd[d]	–
	Carotene a+b		1.7 ± 0.7	1.0 ± 0.3	–
Trace metals	*Fe*	[µg/g dry WE or EAF]	4.4 ± 1.0	85.5 ± 1.2	–
	Cu		0.9 ± 0.0	1.6 ± 0.2	–

[a, b, c] significance between samples in an antioxidant assay at a certain concentration.
[x, y, z] significance between concentration in an antioxidant assay for a specific sample.
[d] nd = not detected or below detection limit.
[e] Radical scavenging activity control = BHT, Metal chelating ability control = EDTA, Reducing power control = Ascorbic acid.

samples prepared with neither fish oil nor WE or EAF. Then, the volatiles were collected in the same way as for the samples.

2.15. Statistics

The results obtained were analysed by two-way ANOVA (GraphPad Prism Version 4.0, GraphPad Software, Inc.). The Bonferroni multiple comparison post-test was used to test difference between samples or storage time. The significance of the results is expressed as having a p-value ≤ 0.05.

3. Results and discussion

3.1. Extract and fraction characterisation

3.1.1. Total phenolic content and bioactive co-extracted compounds

The phlorotannins are the major polyphenolic group in *F. vesiculosus*. The aim of the extraction process was to extract high amounts of phlorotannins due to their known high antioxidant activity. Furthermore, fractionation with ethyl acetate was done to purify the phlorotannins. However, other compounds may be co-extracted. Therefore, trace metals, proteins,

tocopherol and pigments, which may work as either antioxidants or prooxidants were also determined.

In this case phlorotannins were determined as TPC using gallic acid equivalent and not identified individually. TPC of WE and EAF were 18.4 ± 0.1 and 26.5 ± 1.2 g GAE/100 g dry WE or EAF, respectively (Table 1). Wang et al. (2010) and Farvin and Jacobsen (2013) found a higher TPC in extracts where more polar solvents than water had been used for extraction. Nevertheless, Wang et al. (2010) found higher TPC in water extract, 26.3 ± 0.2 of PGEs/100 g extract than in the present study. Also, Wang et al. (2012) got a higher TPC of the ethyl acetate fraction; 88.3 ± 2.2 of PGEs/100 g in ethyl acetate fraction from 80% ethanol *F. vesiculosus* extract. The difference in TPC between our results and results from Wang et al. (2010, 2012) can be due to seasonal variations. TPC is typically higher in the summer and early autumn (Plouguerné et al., 2006).

No detectable levels of proteins were found in WE nor in EAF (data not shown). Holdt and Kraan (2011) stated in a review of nutritional values of seaweed that the protein fraction of seaweed generally is smaller in brown seaweed compared to red and green seaweed. *Fucus* sp. has a maximum content of 17–21% whereas red and green seaweed can have up to 44% protein. Dawczynski, Schubert, and Jahreis (2007) detected both protein and amino acids in brown seaweed. However, these results are based on the protein content of the whole seaweed

and not on extracts or fractions. It can therefore be concluded that the extraction methods did not extract protein in significant amounts.

Both WE and EAF contained α-, β-, γ- and δ-tocopherols as shown in Table 1. While EAF had the highest content of δ-tocopherol, WE contained more α-tocopherol than EAF. Farvin and Jacobsen (2013) also found that *Fucus* sp. contained all four tocopherol homologues. δ-tocopherol has higher antioxidant activity than α-tocopherol in bulk oil (Decker, 1998).

The pigments identified and quantified in the WE and EAF belong to the main groups of carotenoids or chlorophylls. The most abundant carotenoids and chlorophylls found in WE and EAF are shown in Table 1. EAF contained a greater variety of pigments and also much larger amounts than WE. Prasinoxanthin and carotene a + b were the two pigments present in the largest amount in WE (1.3 ± 0.0 and 1.7 ± 0.7 mg/mg dry WE, respectively). In EAF, 19-But-Fuco-xanthin was the major pigment present (43.0 ± 1.9 mg/mg dry EAF), but also chlorophyll c2 was present in large amounts (15.8 ± 2.6 mg/mg dry EAF). Nygård and Ekelund (2006) found that *F. vesiculosus* contained 0.2 μg/mg dw β-carotene, 1 μg/mg dw fucoxanthin and 5 μg/mg dw chlorophyll a. These values were much lower than we found. Though, it should be considered that the results from Nygård and Ekelund (2006) were obtained on whole seaweed and not on extracts as in the present study. Chlorophylls as well as oxidised or breakdown products hereof can have severe pro-oxidative effects, since they can work as sensitisers in photooxidation and formation of singlet oxygen (Andersen & Skibsted, 2010). The amount of carotenoids like fucoxanthin and lutein was especially high in EAF compared with in WE. Fucoxanthin and lutein have been recognised as antioxidants in autoxidation due to their high radical scavenging activity (Le Tutour et al., 1998; Sindhu, Preethi, & Kuttan, 2010). Furthermore, Haila, Lievonen, and Heinonen (1996) found that a combination of lutein and γ-tocopherol was synergistic in inhibiting autoxidation of triacylglycerides.

The main trace metal in WE and EAF was found to be iron (Table 1). The iron content was highest in EAF (85.5 ± 1.2 μg Fe/g dry EAF vs 4.4 ± 1.0 μg Fe/g dry WE). Likewise Cu content was also highest in EAF. Ensminger, Ensminger, Konlande, and Robson (1995) also found seaweed to be a source of trace metals such as Cu, Zn and Fe. Nevertheless the metal content of *F. vesiculosus* has been found to vary significantly between samples harvested at different places and to be season dependent (Phillips, 1979). Thus, Phillips (1979) found the variation in the Øresund region between Denmark and Sweden to be from 53 to 151 μg Fe/g dw *F. vesiculosus*. The Fe content of EAF was within this range. For WE the Fe content was not within the range. Smith, Haan, and Harwood (1986) found that *Fucus* sp could store high amounts of Cu, indicating a high Cu tolerance. Based on their findings they suggested that the high tolerance was due to chelation of the metals in *Fucus* sp. The problem with the presence of trace metals in WE and EAF is their potential prooxidative activity.

In summary, phenolic compounds were extracted to a great extent by the extraction method used. However, large amounts of pigments and trace metals were co-extracted. The fraction of ethyl acetate extracted more pigments, tocopherol and trace metals. The presence of co-extracted pigments might influence the antioxidant activity depending on the storage conditions. The extraction method used was not able to extract protein and amino acids.

3.1.2.   Antioxidant properties

Three different antioxidant assays were used in this study to take into account different antioxidant properties of the WE and EAF. The WE and EAF were tested in the assays in different concentrations (1.0, 1.5 and 2.0 mg dry WE or EAF or Control/mL methanol) to investigate if there was any effect of concentration. Also WE and EAF were compared with commonly used antioxidants such as BHT, EDTA and ascorbic acid. The results are shown in Table 1.

The results were in agreement with previous studies on *in vitro* antioxidant properties of seaweed extracts (Farvin & Jacobsen, 2013; Wang et al., 2009, 2012). Wang et al. (2009, 2012) found a correlation between high phlorotannin content and high radical scavenging activity. Similar relationship was also found in the present study, since the EAF had the highest phenolic content and the highest radical scavenging capacity. Furthermore, the high content of the pigment, fucoxanthin can also contribute to the high radical scavenging activity of EAF. No relationship between increasing radical scavenging activity and increasing concentration was found for neither WE, EAF nor BHT. Nevertheless the reason why concentration dependence was not found could be that the maximum scavenging capacity was reached already at the lowest concentration.

Wang et al. (2009) and Farvin and Jacobsen (2013) also found higher metal chelating ability of water extracts compared with other extracts (ethanol and acetone extracts) as was also observed in the present study. Highly polar compounds, other than phenolic compounds, with metal chelating ability must therefore be co-extracted by water and contribute to the metal chelating ability. Toth and Pavia (2000) reported that compounds such as sulphated polysaccharides (fucoidans) and/or phytochelatins were more effective than phlorotannins for the detoxification to copper accumulation in brown alga *Ascophyllum nodosum*, indicating that these compounds can chelate metals.

The high reducing power of *Fucus* sp. was reported to be due to the presence of fucoidans (Rupérez, Ahrazem, & Leal, 2002). Both WE and EAF were able to reduce Fe^{3+} to Fe^{2+} to the same extent as ascorbic acid. No correlation between concentration and reducing power was found for WE, EAF or ascorbic acid. It has previously been found that ethanol extracts from *Fucus* sp. exhibited lower reducing power than water extract, indicating that water extract contains compounds which contribute to the reducing capacity of the extracts (Farvin & Jacobsen, 2013) such as fucoidans. Since the fucoidans are water-soluble the content in the WE is presumably higher in the WE explaining the higher metal chelating ability and reducing power of this extract compared with the EAF.

3.2.   Oxidative stability in fish-oil-enriched foods with WE and EAF

The droplet size distributions were measured for both milk emulsions and mayonnaise. However, no changes in droplet size during storage were detected. Therefore these results are not included.

Table 2 – The Relative Decrease in Area% (RDA%) of EPA and DHA from day 0 to the end of storage of fish-oil-enriched milk emulsions or mayonnaise with WE and EAF including a control without WE or EAF.

	RDA% EPA	RDA% DHA
Milk_Control	3.9	5.3
Milk_WE1	1.0	1.4
Milk_WE2	1.0	1.5
Milk_WE3	0.9	1.3
Milk_EAF1	0.5	1.1
Milk_EAF2	0.3	0.6
Milk_EAF3	0.4	0.5
Mayo_Control	6.6	15.0
Mayo_WE1	12.4	16.8
Mayo_WE2	6.5	9.2
Mayo_WE3	1.8	3.0
Mayo_EAF1	3.6	4.9
Mayo_EAF2	0.6	2.5

3.2.1. Milk

3.2.1.1. *Changes in EPA and DHA during storage.* The content of EPA and DHA of the total fatty acid composition were determined at day 0 and day 12. *The Relative Decrease in Area%* (RDA%) was calculated to find the changes in content of EPA and DHA during storage (Table 2).

The results clearly showed that lipid oxidation in the milk influenced the content of EPA and DHA. However, a significant reduction in RDA% was observed when WE and EAF was added to the milk emulsions.

Sørensen et al. (2014) found that the content of EPA and DHA ranged between 3.2 and 4.2% in fish-oil-enriched milk. In contrast to the present study, they found no significant changes in EPA and DHA during storage.

3.2.1.2. *Tocopherol content during storage.* Three tocopherol homologues were found in the milk emulsions, α-, β- and δ-tocopherol. Alpha-tocopherol was found in the highest amount ranging from 0.6 to 1.3 μg/g milk emulsion at day 0 (Fig. 1). Nevertheless the total tocopherol content (TTC) was calculated since the changes in the three homologues followed the same pattern during storage. At day 0 there was a significantly lower TTC in the control milk compared to milk with WE or EAF added in all concentrations. After 6 days of storage the TTC of the control milk emulsion was significantly lower than at day 0. After 12 days also milk with the lowest concentration of WE also had a significantly lower TTC than at day 0.

Sørensen et al. (2014) observed similar tocopherol homologues in fish-oil-enriched milk emulsions and found α-tocopherol concentrations in more or less the same range as us. Alemán et al. (2015) observed a decrease in α-tocopherol concentration in fish-oil-enriched milk emulsions without added antioxidants during storage. In the present study, a reduction in tocopherol in the control milk emulsions was also observed while no reduction in TTC was observed for milk emulsions with WE or EAF, except WE in the lowest concentration. This can be due to synergistic relationships between phenolic compounds or pigments and tocopherol in WE and EAF.

WE and EAF contained tocopherol which corresponds to a general higher content of tocopherol in milk emulsions with the WE or EAF, particularly EAF.

3.2.1.3. *Primary oxidation products during storage.* The results of PV are shown in Fig. 2a. The initial concentration of PV varied between milk emulsions at day 0. A significant increase ($P < 0.01$) in PV in milk emulsions with the highest concentration of WE was found from day 0 to 3. No significant increase was found in the other milk emulsions from day 0 to day 3. Hence, the
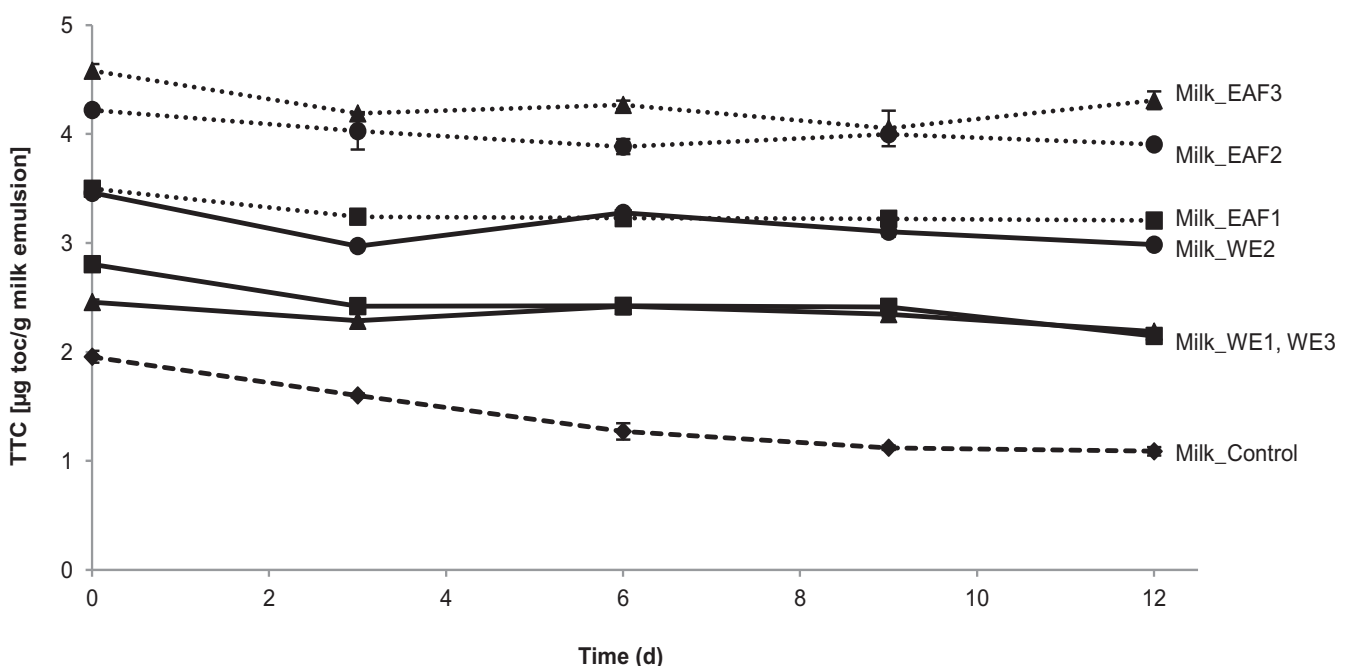
Fig. 1 – Total tocopherol content (TTC) [μg/g milk emulsion] in fish-oil-enriched milk emulsions with and WE or EAF including a control without WE or EAF during storage at 5 °C. Error bars indicate SD of the measurements (n = 2).
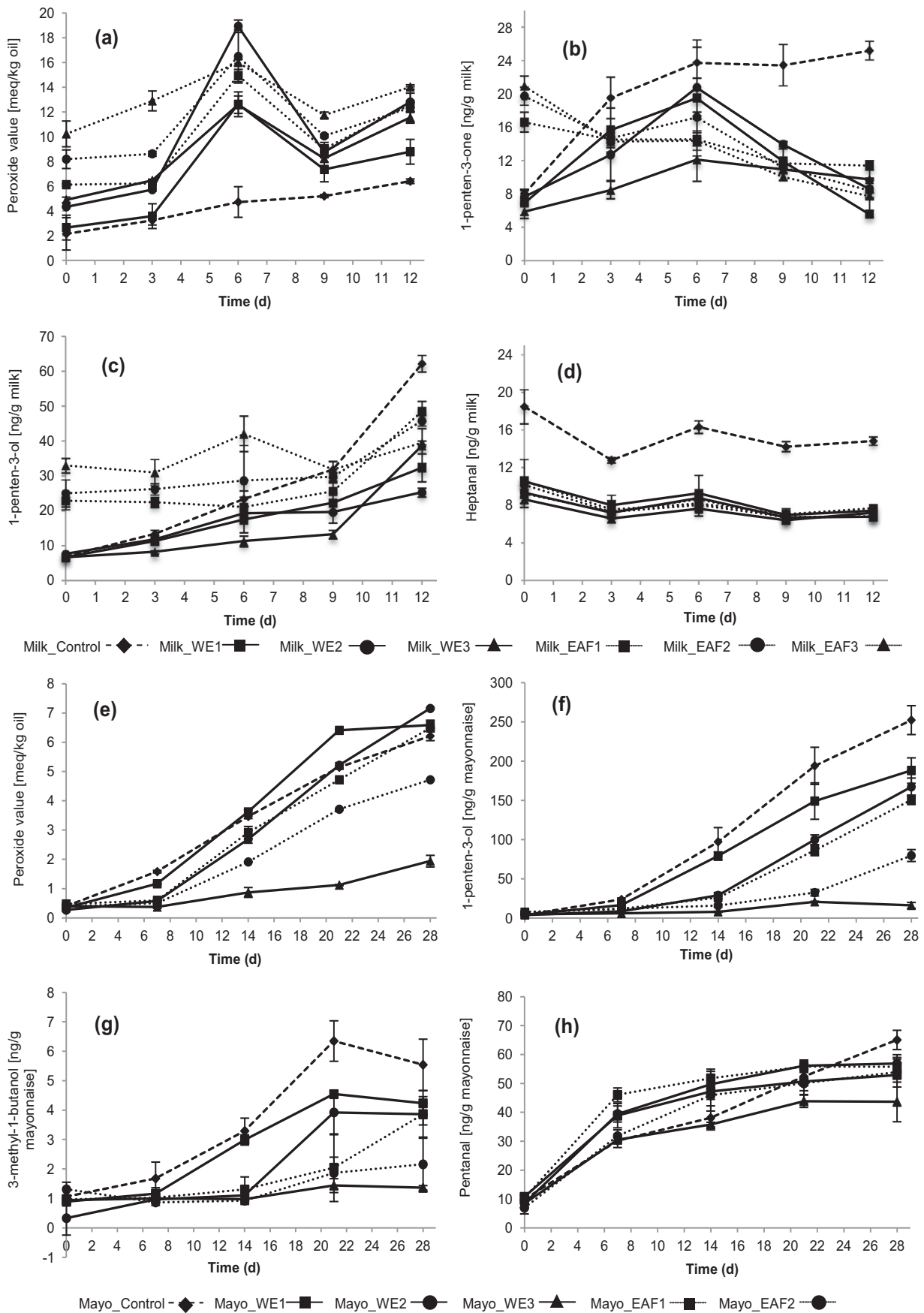
Fig. 2 – Milk emulsions and mayonnaise. Concentration of peroxides measured as PV [meq. peroxides/kg oil] (a and e). Development of secondary oxidation products [ng/g milk or mayonnaise]. Milk emulsions: Concentration of 1-penten-3-one (b), 1-penten-3-ol (c) and heptanal (d) during storage at 5 °C. Mayonnaise: Concentration of 1-penten-3-ol (f), 3-methyl-1-butanol (g) and pentanal (h) during storage at 20 °C. Error bars indicate SD of the measurements (n = 2 for PV and n = 3 for volatiles compounds).

lag phase for these emulsions was three days. After six days of storage, the milk emulsions with WE or EAF had reached maximum PV. Milk emulsions with WE2 had the highest concentration of PV at day 6 (18.92 ± 0.50 meq O_2/kg milk). All seaweed extract or fraction treated milk samples followed the same pattern, where concentration of PV decreased drastically, especially in milk with WE, after day 6. However the reference had significantly lower PV compared to seaweed treated samples throughout the storage time. This could indicate the presence of some compounds which interacted with the method for PV determination.

Sørensen et al. (2014) also found a decrease in concentration of PV in fish oil enriched milk, though this was after 9 days of storage.

The decrease in PV after day 6 in milk emulsions with WE and EAF might be due to formation of secondary oxidation product from primary oxidation products. This can be caused by the presence of reductants or prooxidative activity of the compounds in WE or EAF. Reductants cause the reduction of the Fe^{3+} to the more reactive ferrous form and will increase degradation of peroxides.

3.2.1.4. *Secondary oxidation products during storage.* Nine volatiles in the milk emulsions were identified and quantified. The volatiles were 1-penten-3-ol, 1-penten-3-one, hexanal, heptanal, octanal, nonanal, 2-hexanal, 2-butenal and 2,4-heptadienal. These compounds were selected since they are known as degradation products formed from oxidation of milk or marine lipids. Among others, 1-penten-3-one has been recognised as decomposition products of EPA and DHA (Venkateshwarlu, Let, Meyer, & Jacobsen, 2004a). In addition, 1-penten-3-one has been suggested as one of the markers for fishy and metallic off-flavours in fish-oil-enriched foods (Venkateshwarlu, Let, Meyer, & Jacobsen, 2004b). 1-Penten-3-one together with 1-penten-3-ol and heptanal represented the patterns of the other volatiles that were quantified.

The initial concentration of 1-penten-3-one and 1-penten-3-ol (Fig. 2b and c) differed between samples. Milk emulsions with EAF had significantly higher initial concentration of these two volatiles than the control, whereas milk emulsions with WE had the same initial concentration as the control.

The concentration of 1-penten-3-one increased in the control and milk emulsions with WE from storage day 0 to storage day 6 (Fig. 2b). Hereafter the concentration decreased in milk emulsions with WE, whereas no further increase or decrease in concentration was observed for the control. The concentration of 1-penten-3-one decreased in milk emulsions with EAF throughout the storage. At day 12 all milk emulsions with WE or EAF reached the same 1-penten-3-one level, whereas the concentration of 1-penten-3-one in the control was significantly higher than all the other milk emulsions. This was also detected in sensory evaluation (data not shown). Here, fishy-off odours related to oxidation of fish oil were by the end of the storage detected to a larger extent in control milk emulsion than in milk emulsions containing WE or EAF.

Increasing concentrations of 1-penten-3-one in fish-oil-enriched milk emulsions followed by a decline have been observed before (Alemán et al., 2015; Let et al., 2003; Sørensen et al., 2012). It was suggested to be related to a reduction of this volatile to 1-penten-3-ol.

For development of concentrations of 1-penten-3-ol (Fig. 2c) a lag phase of 3 days was observed before a significant increase in concentration of 1-penten-3-ol in the control and milk emulsions with WE1, WE2 and EAF2. A lag phase of 9 days was observed for milk emulsions with WE3 and EAF1. At day 12 the concentration of 1-penten-3-ol in milk with EAF3 was still not significantly different from that at day 0. However, since the initial concentration of 1-penten-3-ol in milk with EAF was higher than milk with WE, the concentration of 1-penten-3-ol in milk with EAF was higher at day 12 compared with milk with WE. At day 12 the concentration of 1-penten-3-ol in the control was significantly higher than all milk emulsions with WE or EAF. The concentration of 1-penten-3-ol in milk emulsions added EAF2 decreased after day 6.

A lag phase before increase of 1-penten-3-ol in milk emulsions have been observed before (Let et al., 2003; Sørensen et al., 2012). As mentioned the decline in 1-penten-3-one can be related to reduction of this volatile to 1-penten-3-ol. This reduction is indicated by lag phases and a quick increase in concentration of 1-penten-3-ol in the milk emulsions after the lag phases. Nevertheless, this was not observed in milk with EAF3.

The development of 2-butenal and 2-hexenal in the milk emulsions was similar to what was observed for 1-penten-3-ol. Sørensen et al. (2014) also found that development of 2-hexenal and 1-penten-3-ol was similar in fish-oil-enriched milk.

The concentration of heptanal in the milk emulsions is shown in Fig. 2d. Initially the concentration of heptanal was significantly higher in the control than in the other milk emulsions. This higher concentration in the control was observed throughout the storage period. No significant increase in the concentration of heptanal was observed in the control and in the milk emulsions with WE or EAF during storage.

Like heptanal the development of hexanal, nonanal and octanal are also related to the oxidation of milk (data not shown). Nonanal and octanal followed the same pattern as was found for heptanal, where no significant increase in concentrations was found over storage. In contrast, hexanal followed the same pattern as 1-penten-3-one except from the decline in the control and milk emulsions with WE. No 2,4-heptadienal was detected in milk emulsions with WE or EAF. However, a precipitous increase in concentration of 2,4-heptadienal in the control was observed at storage day 9.

Due to the fact that PV and concentrations of some of the volatiles were higher at day 0 in samples with EAF it was difficult to interpret the findings for these samples. Nevertheless, EAF did show antioxidant activity since the reduction in content of EPA and DHA was lower in milk emulsions with WE or EAF compared to the control, where EAF was the most efficient to lower the decomposition of EPA and DHA. This was confirmed by the development of EPA and DHA decomposition compound, 1-penten-3-one, where milk emulsions with WE or EAF either had slower development or decrease of 1-penten-3-one compared to the control. Furthermore, extended lag phases in development of 1-penten-3-ol were observed for milk with EAF1 and EAF3 compared to the control milk emulsion indicating an antioxidant activity.

3.2.2. Mayonnaise

3.2.2.1. Changes in EPA and DHA content during storage. In mayonnaise with WE or EAF the RDA% of EPA and DHA is shown in Table 2. A minimisation of RDA% of EPA and DHA was found in mayonnaise when EAF was added. The highest concentration of EAF was most efficient to decrease the RDA% of EPA and DHA. In mayonnaise with WE only the highest concentration decreased the RDA% of both EPA and DHA. At the lowest concentration WE showed prooxidant activity towards oxidation of EPA and DHA in the mayonnaise since the RDA% of both EPA and DHA was higher in mayonnaise with WE1 than the control.

In contrast to our findings, Alemán et al. (2015) did not find indications that EPA and DHA were oxidised in mayonnaise during storage period of 28 days when evaluating antioxidant efficacy of lipophilised caffeic acid.

3.2.2.2. Tocopherol content during storage. All four tocopherol homologues were detected in the mayonnaise. α-tocopherol was the only homologue where the development from day 0 to 28 was significant between samples (data not shown). Therefore the content of α-tocopherol is stated in Table 3.

The content of α-tocopherol was the same in all mayonnaise at day 0. However at day 28 the content of α-tocopherol in the control mayonnaise had decreased more compared to mayonnaise with WE3 and EAF2.

Degradation of α-tocopherol in mayonnaise during storage of 28 days was also found by Alemán et al. (2015).

The higher decrease of α-tocopherol in the control mayonnaise indicates that oxidation was more distinct in this sample and that α-tocopherol was used as antioxidant.

As mentioned *F. vesiculosus* also contained α-, β-, γ- and δ-tocopherols. However, contradictory to the milk emulsions, the tocopherol contribution of the WE and EAF was unimportant in the mayonnaise since the contribution of tocopherols from rapeseed oil was much larger than that from WE and EAF.

3.2.2.3. Primary oxidation products during storage. The results for the development in PV during storage are shown in Fig. 2e. A lag phase was observed until day 7 for mayonnaise with WE and EAF, except for mayonnaise with WE1. No lag phase was observed for the control mayonnaise. At day 14 PV in all mayonnaise samples with WE or EAF had increased significantly from day 0. This increase continued throughout the storage period. At the end of storage, PV in all mayonnaise samples was significantly higher than at day 0. Mayonnaises with WE3 or EAF2 were found to have significantly lower PV than all the other mayonnaises. The final PV of mayonnaise with WE3 and EAF2 were 1.9 ± 0.2 and 4.7 ± 0.0 meq O_2/kg oil, respectively. At day 28, mayonnaise with WE1 had the highest PV content of all mayonnaises (7.1 ± 0.0 meq O_2/kg oil) and thus showed prooxidative behaviour.

Prooxidant activity of WE1 was also observed in the oxidation of EPA and DHA. However, before clear conclusion can be drawn, date on volatile oxidation products must be considered.

3.2.2.4. Secondary oxidation products. Eight volatiles were identified and quantified, namely 1-penten-3-ol, 1-penten-3-one, 3-methyl-1-butanol, 1-pentanol, hexanal, nonanal, pentanal, 2,4-heptadienal. These compounds have also been identified in fish-oil-enriched mayonnaise by Hartvigsen et al. (2000).

Fig. 2f–h shows the development in 1-penten-3-ol, 3-methyl-1-butanol and pentanal. Hexanal, 2,4-heptadienal, 1-pentanol, 1-penten-3-one and 4-methyl-1-pentanol followed similar pattern (data not shown) and no significant increase was observed in the concentrations of these compounds during storage.

Development of 1-penten-3-ol (Fig. 2f) was induced by a lag phase for all mayonnaise samples. For the control and mayonnaise with WE1 and WE2, the lag phase was 7 days. The lag phase of mayonnaise with EAF in both concentrations was 14 days. At the end of the storage period the concentration of 1-penten-3-ol in mayonnaise with WE3 had still not increased significantly from day 0.

As already mentioned a lag phase of 1-penten-3-ol are common since 1-penten-3-ol can be the reduction product of 1-penten-3-one. However, no significant decline in 1-penten-3-one as the concentration of 1-penten-3-ol increased was observed (data not shown).

Similar to the observations for 1-penten-3-ol, a lag phase was also observed for 3-methyl-1-butanol (Fig. 2g). For the control and mayonnaise with WE1, the lag phase was 7 days. The lag phase of 3-methyl-1-butanol in mayonnaise with WE2 and EAF1 was found to be 14 days. At the end of the storage period the concentration of 3-methyl-1-butanol in mayonnaise with WE3 and EAF3 had still not increased significantly from day 0.

Development of pentanal (Fig. 2h) in the mayonnaise samples was different from 1-penten-3-ol and 3-methyl-1-butanol. The concentrations increased significantly in all mayonnaise samples from day 0 to day 7. Thereby no lag phases were observed. However, after day 7 the development in pentanal in the mayonnaise samples evened out. Thus, no further increase of pentanal in mayonnaise with WE or EAF was observed after day 21.

In summary, the antioxidant activity of WE or EAF was much clearer in mayonnaise than in milk. WE3 and EAF in all concentrations showed great potential to lower the degradation of EPA and DHA. WE3 was found to be most efficient in decreasing development of PV and secondary oxidation products followed by EAF2. Prooxidant activity of WE1 was observed on the degradation of EPA and DHA. Furthermore, indication of no or prooxidative activity of WE1 was found in a sensory

Table 3 – α-tocopherol content (µg toc/g oil) (mean ± SD, n = 2) in fish-oil-enriched mayonnaise at day 0 and 28 including a control without WE or EAF.

| | α-tocopherol | |
| | [µg toc/g oil] | |
	Day 0	Day 28
Mayo_Control	192.81 ± 14.09	177.46 ± 0.29
Mayo_WE1	195.24 ± 3.44	189.84 ± 2.07
Mayo_WE2	192.54 ± 1.70	184.10 ± 0.25
Mayo_WE3	190.63 ± 0.83	193.69 ± 0.93
Mayo_EAF1	193.95 ± 5.66	188.99 ± 0.41
Mayo_EAF2	191.92 ± 8.33	195.56 ± 2.05

evaluation on development of fishy off-flavour in the mayonnaise samples during storage (data not shown). It was found that the biggest increase in fishy off-flavour from day 0 to day 28 was observed in the control and mayonnaise with WE. This indicated that oxidation occurred to the same or higher extents in mayonnaise with WE1 as in the control. Thus, according to the results of secondary oxidation products, mayonnaise with WE1 and the control had the highest content of fishy-off flavour related to the content of 1-penten-3-ol.

4. Efficacy of WE and EAF to decrease lipid oxidation in fish-oil-enriched foods

The compositional data of WE and EAF indicated that the extraction and fractionation method used could extract phenolic compounds. The co-extracted compounds were mainly iron, tocopherol and pigments. The antioxidant activity of WE and EAF could not only be associated with phenolic compounds since pigments were present to a larger extent compared to the concentration of phlorotannins as analysed by TPC. Extraction first with 80% ethanol and fractionation with ethyl acetate yielded also high amounts of trace metals, especially of iron. Nevertheless, the high iron content of EAF was not found to negatively influence the oxidative stability of milk emulsions or mayonnaise since EAF did not show prooxidant activity in the foods. This could be due to the presence of the high amount of phenolic compounds in EAF including phlorotannins which are able to chelate metals disabling them to participate in lipid oxidation.

High radical scavenging activity was found to be the most important antioxidant property to inhibit lipid oxidation in fish-oil-enriched milk. Thus, EAF, which had higher radical scavenging activity than WE, decreased the degradation of EPA and DHA to a larger extent than WE. Furthermore, EAF reduced the formation of secondary EPA and DHA degradation products more efficiently than WE and decreased the formation of secondary oxidation products related to oxidation of fish oil. The high radical scavenging of EAF can be associated with TPC and pigment content, which was much higher in this fraction compared with WE. However, in the perspective of commercialised foods with EAF as antioxidant, a high content of pigments like chlorophyll can be detrimental to the oxidative stability.

High metal chelating ability has been found to be a crucial antioxidant property in order for antioxidants to work efficiently in mayonnaise. WE exhibited the highest metal chelating ability compared to EAF. The metal chelating ability of WE was considered to be related to other highly polar compounds than phenolic compounds, but this has to be further investigated. EAF was found to be more efficient in decreasing the degradation of EPA and DHA than WE. However, WE3 in mayonnaise was found to lower the formation of PV and inhibit the formation of 3-methyl-1-butanol and 1-penten-3-ol completely. The efficacy of WE was found to be depending on concentration since both the degradation of EPA and DHA and the formation of PV was higher in mayonnaise with the lowest concentration compared with the control.

Likewise, Wang et al. (2010) evaluated the antioxidant activity of *F. vesiculosus* extracts and fractions in cod muscle and cod protein isolates. They found that phlorotannin-enriched ethyl acetate fraction showed higher inhibitory effect than crude 80% ethanol extract on development of rancid odour in cod.

All in all, application of *F. vesiculosus* extracts and fractions in foods should be further investigated. This includes identification and characterisation of phlorotannins and determining how these polyphenolic compounds contributes to the overall antioxidative activity of the extracts.

5. Conclusion

The efficacy of *F. vesiculosus* water extract and ethyl acetate fraction as antioxidants in foods differed depending on food system. EAF in a concentration of 2 g/kg was most efficient as antioxidant in milk emulsions. In mayonnaise, EAF in concentration of 2 g/kg was most efficient with respect to decreasing the degradation of the healthy n-3 PUFA. However, WE in concentration of 2 g/kg was more efficient in decreasing formation of PV and volatiles. Based on the findings, high antioxidant efficacy of WE and EAF in fish-oil-enriched foods was first of all concentration dependent. Moreover, the efficacy of WE and EAF was related to high TPC, high radical scavenging activity, moderate or high metal chelating ability and high carotenoid content.

Acknowledgements

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PAPER II

Potential seaweed based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise

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CrossMark

Potential seaweed-based food ingredients to inhibit lipid oxidation in fish-oil-enriched mayonnaise

Philipp J. Honold[1] · Charlotte Jacobsen[1] · Rósa Jónsdóttir[2] · Hordur G. Kristinsson[2] · Ditte B. Hermund[1]

Abstract Brown seaweed *Fucus vesiculosus* has a high potential as a source of natural antioxidants due to a high diversity of bioactive compounds in its composition. In this study, four extracts were characterized with respect to composition of bioactive compounds, in vitro antioxidant properties and their partitioning between water and octanol. Additionally, the antioxidant activity of the extracts was evaluated in a fish-oil-enriched mayonnaise. Acetone and ethanol were found to extract the highest amount of phenolic compounds and carotenoids. Water used as extraction solvent, extracted some phenolic compounds but also higher amount of metals and chlorophyll derivates. It was proposed that extracts with high phenolic content and low iron content, such as the acetone and ethanol extract, would have the highest potential as antioxidants in foods. This was confirmed in the storage trial, where these extracts showed higher antioxidant activity.

Keywords Brown algae · Phlorotannins · n-3 LC-PUFA · Pigments · Food emulsions · Lipid oxidation · Mayonnaise · *Fucus vesiculosus*

Abbreviations

AcE	Acetone extract from *F. vesiculosus*
DHA	Docosahexaenoic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EPA	Eicosapentaenoic acid
EtE	Ethanol extract from *F. vesiculosus*
F. vesiculosus	*Fucus vesiculosus*
GC	Gas chromatography
HPLC	High-pressure liquid chromatography
LC-PUFA	Long-chain polyunsaturated fatty acids
logP	Octanol/water partitioning coefficient
o/w emulsion	Oil-in-water emulsion
PCA	Principal component analysis
PV	Peroxide value
TPC	Total phlorotannin content
WoE	Aqueous extract from old parts of the *F. vesiculosus* leaf
WyE	Aqueous extract from young parts of the *F. vesiculosus* leaf

Introduction

Fish oil is rich in omega-3 LC-PUFA, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) which are beneficial to human health [1, 2]. Due to increased consumer awareness of the beneficial effects of DHA and EPA in human nutrition, the demand for functional foods enriched with LC-PUFA of marine origin is increasing. However, fortified foods with omega-3 LC-PUFA-rich fish oil shows a decrease in oxidative stability. This leads to the development of undesirable off-flavours and reduced shelf life [3]. Therefore, it is necessary to increase the shelf life of these products and sustain consumer acceptance.

Antioxidants are used to enhance the oxidative stability of lipid-rich food. Synthetic antioxidants are widely used for this purpose, e.g. butylated hydroxytolulene (BHT) and ethylenediaminetetraacetic acid (EDTA) because they are cheap and easy to use. However, synthetic antioxidants can have negative effects on human health, so the use of

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these antioxidants is restricted by regulations in order to minimize health risks [4]. Due to these reasons, there is an interest in natural alternatives.

Many natural antioxidants of plant or marine algae origin show great potential for improving oxidative stability of food, and additionally, it has been shown that these natural antioxidants have a broad array of health-promoting benefits [5, 6]. Seaweed contains several bioactive compounds, such as pigments (carotenoids, chlorophylls), different tocopherols, sulphated polysaccharides (fucoidans), amino acids and mono- and polyphenols [7–10]. All these bioactive compounds can influence the antioxidative properties of seaweed or extracts hereof. Another factor is the difference in biochemical composition of different parts of the seaweed. Growing or young parts of the algae are located at the leaf tips and are easily recognized by their bright green colour and have a different composition of bioactive compounds compared to older parts. The growing parts are enriched with substances which are necessary for building new cellular structures [11]. The obvious divergence in colour is an indication that the composition, mainly the pigment composition in the young parts of the seaweed leave, is different compared to the older parts of the leaf.

Fucus vesiculosus belongs to the group of brown algae. Beside a broad array of bioactive compounds like pigments, *F. vesiculosus* is known to produce high amounts of polyphenolic secondary metabolites called phlorotannins [12, 13]. These phlorotannins have been found to be the main contributors to the antioxidant activity of brown algae extracts [7, 12–14]. The high antioxidant activity of phlorotannins is due to their chemical structure. Phlorotannins consist of up to eight interconnected rings (phloroglucinol units), which makes phlorotannins more effective in preventing oxidation than monophenols and polyphenols with less phenolic rings due to the higher number of functional –OH groups [12]. Whereas the antioxidant properties of *F. vesiculosus* have been widely studied in simple in vitro assays [7, 12, 13], the knowledge on application of *F. vesiculosus* extracts in foods is sparse [15, 16].

Ethyl acetate is known to extract polyphenolic compound to a high extent [15]. But little is known about the extraction yield of other bioactive compounds. Furthermore, extracting with ethyl acetate is costly and time-consuming and the food safety of the final product could be impacted. Therefore, less harmful extraction solvents like water and ethanol were used in this study. Acetone was included as well since the extraction with acetone is easier and leads to a high yield of whole extract which makes it preferable over ethyl acetate in the industry. Due to this reasons, water, ethanol and acetone were evaluated as extraction solvents in this study.

The efficacy of these types of extracts in food emulsions not only depends on the composition but also on the availability of them at the place where the oxidation occurs. Mayonnaise is an oil-in-water (o/w) emulsion, in which lipid oxidation normally is initiated at the interface between oil and water and then progresses in the oil phase. Antioxidants that are located at or close to the interface normally also show high antioxidant efficacy [17].

Besides that, other factors are influencing the oxidative stability in mayonnaise. First, the amount of unsaturated fatty (or acyl) chains contributes to a high extent to the oxidative instability [18]. The presence of trace metals plays a major role in lipid oxidation as well. Trace metals such as iron can promote reactions with unsaturated lipids to form lipid radicals (alkyl radicals) or lead to degradation of peroxides to alkoxyl radicals. In most emulsified foods, trace metals are naturally present in the water phase. In mayonnaise, a big proportion of the iron present stems from egg yolk, which is traditionally used as the emulsifying agent. The major amount of iron in native egg yolk is bound to the protein phosvitin [19]. However, when the egg yolk is used as an emulsifier in mayonnaise, the low pH of mayonnaise (pH 4) causes breaking of the iron bridges and releasing of iron. This enables it to participate in lipid oxidation [20].

Antioxidants should have good free radical scavenging properties to prevent initiation of lipid oxidation. Scavenging of pre-existing free radicals and radicals which are formed during storage is crucial to their ability to limit lipid oxidation. Furthermore, the ability to chelate trace metal is an important antioxidant property, especially in food emulsions with low pH. It has been found that the most efficient inhibitor of metal-catalysed oxidation in fish-oil-enriched mayonnaise is the synthetic antioxidant EDTA [21]. This is due to its ability to capture metals and prevent them to participate in the metal-catalysed degradation of peroxides.

The aim of this study was to determine the antioxidant properties of four extracts from Icelandic brown algae *F. vesiculosus* and to relate these properties to their composition of bioactive compounds. Bioactive compounds and oxidation products were characterized and quantified with different spectrophotometric methods and HPLC. Furthermore, the antioxidant efficacy of the extracts was evaluated in fish-oil-enriched mayonnaise.

Materials and methods

Materials

Ingredients for mayonnaise like salt and sugar were purchased from the local supermarket. Whole egg powder was purchased from Sanovo foods (Odense, Denmark). Yellow colour (E160a), lemon juice concentrate and Stabilizer Xanthan gum FG (E415) were obtained from Crown Food (Mørkøv, Denmark). Potassium sorbate

(E202) was purchased from Merck (Darmstadt, Germany). Rapeseed oil and fish oil (cod liver) were supplied by Maritex A/S (TINE, BA, Sortland, Norway). The fish oil, used in the mayonnaise preparation, had a PV of <0.1 meq peroxides/kg oil and a tocopherol content of 190 μg α-tocopherol/g oil, 0 μg β-tocopherol/g oil, 85 μg γ-tocopherol/g oil and 35 μg δ-tocopherol/g oil. The fatty acid composition was as follows: 14:0, 3.6 %; 16:0, 9.7 %; 16:1 (n-7), 8.9 %; 18:0, 2.0 %; 18:1 (n-9), 16.4 %; 18:1 (n-7), 4.8 %; 18:2 (n-6), 1.8 %, 20:1 (n-11 + n-9), 12.6 %; 20:5 (n-3) (EPA), 9.4 %; 22:1 (n-11), 5.9 %; 22:5 (n-3), 1.1 % and 22:6 (n-3) (DHA) 11.4 %. Rapeseed oil, used in the mayonnaise preparation, had a PV of 0.25 meq peroxides/kg oil and a tocopherol content of 161 μg α-tocopherol/g oil, 0 μg β-tocopherol/g oil, 251 μg γ-tocopherol/g oil and 6 μg δ-tocopherol/g oil. The fatty acid composition was as follows: 16:0, 4.6 %; 18:0, 1.6 %; 18:1 (n-9), 56.0 %; 18:1 (n-7), 2.7 %; 18:2 (n-6), 20.5 %; 18:3 (n-3), 10.6 %; 20:1 (n-11) + (n-9), 1.3 %.

All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Lactic acid 60 %, acetic acid 20 % and the external standards were purchased from Sigma-Aldrich (Steinheim, Germany).

Extraction and fractionation

Four different extracts of *F. vesiculosus* provided by Matís, Iceland, were used in this study: an acetone extract (AcE), ethanol extract (EtE) and two water extracts, one made from the old parts of the *F. vesiculosus* leaf (WoE) and one from the young parts (WyE). The extraction was carried out by Matís in Iceland according to Wang et al. [12, 13, 16]. The procedure was as follows: freshly collected seaweed was washed with clean sea water to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water and wiped with paper towel. The samples were lyophilized for 72 h, pulverized into powder and stored at −80 °C prior to extraction. Five grams of the algal powder was mixed with either 100 mL of distilled water, 70 % aqueous acetone or 80 % ethanol (v/v) and incubated in a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm at room temperature. The mixtures were centrifuged at 2168g for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. Acetone and ethanol in the solvent extracts were removed by rotary evaporation. The concentrates after rotary evaporation and the supernatant of water extract were freeze-dried. Each extraction was conducted in duplicate. The dried duplicate extracts were pooled and stored at −80 °C until analysed.

Characterization of extracts

Compound characterization

Total phenolic content (Folin–Ciocalteu) In order to estimate the phlorotannins content, the major polyphenolic group in *F. vesiculosus*, the total phenolic content (TPC) of the extracts was determined. The quantification was carried out according to Farvin and Jacobsen [7]. In brief, 100 μL of sample solution (dissolved in methanol, concentration of 1.5 and 2.0 mg/mL) was mixed with 0.75 mL of Folin–Ciocalteu reagent (10 % w/w in distilled water). After 5 min, 0.75 mL of sodium carbonate (7.5 % w/w in distilled water) was added. The samples were incubated for 1.5 h at room temperature in the dark. Sample (200 μL) was transferred to a microtiterplate and measured at 725 nm. The absorbance was measured at 725 nm with an UV–Vis spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). A standard curve from gallic acid was made for calibration (concentrations from 0 to 100 μg/mL). The results are expressed as gallic acid equivalent (GAE) mg/100 g dried extract.

Trace metals Prior to trace metal determination, the extracts were destructed. Briefly, 0.5 g extract with 5 mL HNO$_3$ (68 % v/v), 3 mL H$_2$O$_2$ (30 % v/v) and 0.5 mL HCl (37 % v/v) was mixed and heated up in a destruction oven (Multiwave 3000, Anton Paar, Graz, Austria) for 10 min at 1400 W. Afterwards samples were cooled down to room temperature for 20 min. Samples were diluted with water to reach acidity below 5 % before analysis. Two blank samples without extracts and two reference samples with fish protein certified reference material for trace metals (DORM-3) were prepared (from National Research Council Canada). The analysis was performed using Inductively Coupled Plasma Mass Spectrometry (ICPMS) (ICPMS model 7500ce, Agilent Technologies, Wilmington, Delaware, USA). The instrument was equipped with a Scott-type spray chamber and a concentric nebulizer, and the sample solutions were delivered by a CETAC auto sampler (ASX-500, CETAC Technologies, Omaha, NE, USA). The analysis was done on the isotope 57Fe with the ICPMS in collision–reaction-cell (CRC) mode using helium as cell gas for interference reduction. The calibration was done using external calibration with standard solutions of copper and iron in the concentration range from 0 to 200 μg/L. The analysis was performed in duplicate.

Protein The % protein (w/w) was determined by Kjeldahl method [22]. Seaweed extracts (0.3 g) were weighted with a nitrogen-free weight paper into Kjeldahl tubes. Two Kjeltabs CT (Thompson and Capper, Ltd, England) and 20 mL H$_2$SO$_4$ were added per tube and heated up for 1 h to 440 °C until the samples turned green and kept at

440 °C for 30 min. Then, samples were cooled down. To each tube, 60 mL water was added. Samples were distilled using 2100 Kjeltec distillation unit (Foss, Hillerød, Denmark) (10 × 10 mL 32 % NaOH). The distillate and 20 mL 0.1 M HCl and 5 drops of Andersen Indicator were put into Erlenmeyer flasks. The distillates were titrated with 0.1 M NaOH until indicator turned green (Dosimat 665, Metrohm, Herisau, Switzerland). Average conversion factor used was 5.38 for nitrogen to protein in brown algae [23]. Experiment was performed in duplicate, and two blank samples without algae extract were included.

Pigments The analysis of pigments was based on an HPLC method of Van Heukelem and Thomas [24] with some modifications. In short the extraction procedure was as follows: 10 mg extract and 1 mL acetone were mixed. Sonication was performed for 1 h on ice in the dark. Then, samples were centrifuged for 10 min at 13,684g, and the supernatant was removed. This was then repeated and the supernatants were pooled and filtered with a hydrophobic filter (pore size 0.22 μm) before analysis.

Pigment analysis was performed on an Agilent 1100 Series HPLC (Agilent Technology, CA, USA) equipped with a diode array detector. Separation was obtained on an ZORBAX Eclipse XDB-C8 column from Agilent with a linear gradient consisting of A: methanol and B: buffer (0.028 M tetra butyl ammonium acetate in water, pH = 6.5) starting at 5 % B increasing to 90 % B in 27 min and held for 7 min at 90 %, further increase to 100 % in 1 min where it was held for 3 min, decreasing from 100 to 90 % B in 2 min, returned to 5 % B in 6 min and held there for 2 min. Injection volume was 100 μL, and the flow rate was 1.1 mL/min at 60 °C. The pigments were quantified as equivalents of astaxanthin (carotene) and chlorophyll a (chlorophylls). Calibration curve of astaxanthin for carotenoids and chlorophyll a for chlorophylls was used due to similar response factors of the pigments. Pigments were measured at 450 and 440 nm as detection wavelengths. For the internal standard (tocopherol acetate), the wavelength was 222 nm. Internal standard was used to correct for volume errors due to the volatile acetone used for extraction. Experiment was performed in duplicate, and results were expressed in μg pigments/mg dry weight.

Partitioning coefficient Measurement of the octanol/water partitioning coefficient (logP) is based on the method of the OECD Guidelines for the testing of chemicals (OECD 1995) with some modifications. About 20 mg extract was weighted into 15-mL plastic tubes with cap and 3 mL water filtered with Millipore filter system and 3 mL 1-octanol was added. Headspace was flushed with nitrogen to remove oxygen. All samples were mixed well and put on a shaking plate at 340 rpm in the dark for 18 h. After shaking, the samples were centrifuged for 10 min at 1665g to separate the phases and the remaining amphiphilic layer at the interface. The octanol and water layer were stored at 8 °C until further analysis. The amphiphilic layer between octanol and water was removed, and its TPC content was calculated as the difference between the added amount and the sum of the water and octanol layer.

TPC was then determined in both octanol and water phase according to the method used above with few alterations.

100 μL sample and either 100 μL pure octanol or water were mixed so that all samples contained octanol and water. In 48 % ethanol, 0.7 mL of 5 % Folin–Ciocalteu reagent was added and incubated 5 min at room temperature. After incubation, 0.7 mL 2 % bicarbonate solution in 48 % ethanol was added and incubated for 90 min at room temperature. Thereafter, 200 μL was transferred to a microtiterplate and samples were measured at 725 nm. A calibration curve made with gallic acid was used to determine the concentration in each phase. TPC in each phase was calculated in gallic acid equivalents per 100 g extract (GAE/100 g extract). The analysis was performed in triplicate.

In vitro antioxidant properties

DPPH radical scavenging activity, Fe^{2+} chelating activity and reducing power were tested to characterize the antioxidant properties of the four extracts. All experiments are carried out at concentrations of 1.5 and 2 mg/mL methanol, and the positive control was prepared the same way.

DPPH scavenging activity The radical scavenging ability of the extracts was determined according to the method described by Yang et al. [25] modified using multiplate reader. One hundred and fifty microlitres extract solution was mixed with 150 μL 0.1 mM DPPH (in 96 % ethanol). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma-Aldrich, Steinheim, Germany. Two hundred microlitres of the mixture were added to a microtiterplate, and the absorbance was measured at 20 °C at 517 nm after 30 min (A_s). Triplicate measurements were performed, and BHT in the same concentrations as the samples was included as a positive control. A sample blank was made with DPPH but without extract solution (A_b), and a sample control was made without DPPH but with extract solution (A_0). The results were given as per cent inhibition and were calculated as followed:

$$\text{Inhibition (\%)} = \left(1 - \frac{A_s - A_0}{A_b}\right) \times 100.$$

Fe^{2+} chelating activity The Fe^{2+} chelating activity was determined according to the method described by Farvin et al. [26] with modifications. To the microtiterplate, 100 μL

extract solution and 135 µL distilled water were added. To initiate the reaction, 5 µL 2 mM ferrous chloride was mixed into the solution. After 3 min, 10 µL 5 mM ferrozine was mixed into the solution and the absorbance was measured at 562 nm (A_s). Triplicate measurements were performed, and EDTA in the same concentrations as the samples was included as a positive control. A sample blank was made without extract solution (only iron and ferrozine) (A_b), and a sample control was made with only the extract solution (A_0). The Fe^{2+} chelating activity in per cent was calculated as follows: Fe^{2+} chelating activity $= \left(\frac{A_b - (A_s - A_0)}{A_b} \right) \times 100$.

Reducing power The reducing power was determined according to the method described by Yang et al. [25] with modifications. Two hundred microlitres extract solution was mixed with 200 µL 0.2 M phosphate buffer (pH 6.6) and 200 µL 1 % potassium ferricyanide and incubated in a water bath at 50 °C for 20 min. Two hundred microlitres of the incubated solution was mixed with 10 % TCA. Two hundred and twenty-eight microlitres of this solution was diluted with 228 µL distilled water, and 46 µL 0.1 % ferric chloride was added. After 10 min of incubation at 20 °C, 200 µL of the mixture was added to the microtiter plate and the absorbance was measured at 700 nm (A_s). Triplicate measurements were performed, and ascorbic acid in the same concentrations as the samples was included as a positive control. A sample control was made with only the extract solution (A_0). The results were given in OD_{700} ($A_s - A_0$).

Production of fish-oil-enriched mayonnaise

The mayonnaise was prepared according to Meyer and Jacobsen [27] with minor modifications in the recipe. Each batch was 1000 g, prepared under vacuum using a Stephan Universal mixer (Stephan UMC5, Hameln, Germany). Each batch contained by weight 61.48 % rapeseed oil, 15.37 % fish oil, 18.25 % water, 2.00 % heat stable egg yolk powder, 0.09 % lemon juice concentrate, 1.30 % sugar, 0.60 % salt, 0.005 % yellow colour, 0.08 % lactic acid (80 %), 0.72 % acetic acid, 0.05 % xanthan gum FG and 0.08 % potassium sorbate adding up to approximately 100 %. Egg yolk powder, potassium sorbate, yellow colour, salt, sugar and dry *F. vesiculosus* extract in concentrations from 1.5 g/kg (C1) to 2.0 g/kg (C2) were mixed into the water phase. The water phase was mixed on Stephan mixer for 15 s under vacuum. Rapeseed oil and fish oil were mixed. Some of the oil phase (ca. 5 mL) was taken and mixed with xanthan gum. This mixture was added to the water phase and mixed on Stephan mixer for 15 s under vacuum. To the remaining oil phase, lemon juice, lactic acid and acetic acid were added. The oil phase was slowly poured (2 min duration) into the Stephan mixer without vacuum, to form the mayonnaise. Finally, the mayonnaise was mixed under vacuum for 30 s.

Mayonnaise without extract added was also made following the same procedure (Control).

The mayonnaises were stored in 300-mL brown bottles, at 20 °C for 28 days in the dark. Samples were taken at 7 different time points (days 0, 3, 7, 10, 13, 21 and 28) and stored at −40 °C until analysis.

Lipid phase extraction and oil phase separation

Tocopherol content, fatty acid composition and peroxide value analysis was performed on either extracted lipid phase from the seaweed extracts or on the oil phase, separated from the mayonnaise.

For tocopherol analysis in extracts, the oil was first extracted according to Bligh and Dyer [28]. Fifty milligrams extract was weighted into glass centrifuge tubes with lids, and 1 mL water, 3 mL methanol and 3 mL chloroform were added. Samples were mixed 1 min with Polytron PT 1200E mixer, and afterwards 1.5 mL water was added. Samples were centrifuged at 1665*g* for 10 min and filtered with a hydrophobic phase separation filter. The chloroform was evaporated with nitrogen, and after evaporation oil was resolved in 1 mL heptane that was used for HPLC analysis.

The oil phase from the mayonnaise was extracted by freezing the mayonnaise at −40 °C and after thawing centrifuged for 10 min at 3067*g* to separate the oil and water phase.

Tocopherol content and fatty acid composition of extracted lipid or oil phase

Tocopherol content was determined according to the AOCS official method Ce 8-89 [29].

Alpha, beta, gamma and delta tocopherol homologues were quantified by high-performance liquid chromatography—fluorescent detection (HPLC-FLD).

A standard solution was prepared to determine the retention time of the tocopherols and the peak areas of the given standards. The peak areas of the standard solution were used to calculate the tocopherol content of the samples.

All analyses were done in duplicate. The results were given in µg tocopherol per gram dry extract (µg toc/g dry extract) for the extracts and in µg tocopherol per gram oil (µg toc/g oil) for the oil phase of the mayonnaise.

The fatty acid compositions of the seaweed extracts, the mayonnaise and oil used for the mayonnaise were determined by AOCS official method Ce 1b-89 [30] with modifications using a GC-FID. Toluene and heptane with internal standard (C23:0) (400 µL, 1:3 v/v) were added, and the lipids were methylated in a one-step procedure using a microwave oven (Multiwave3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave were 5 min at 500 W followed by 10 min cooling.

The FAMEs were dissolved in heptane and analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to (AOCS, 1998). For separation DB127-7012 column (10 m × ID 0.1 mm × 0.1 μm film thickness, Agilent Technologies, Palo Alto, CA, USA) was used. Injection volume was 0.2 μL in split mode (1:70). The initial temperature of the GC-oven was 160 °C. The temperature was set to increase gradually as follows: 160–200 °C (10.6 °C/min), 200 °C kept for 0.3 min, 200–220 °C (10.6 °C/min), 220 °C kept for 1 min, 220–240 °C (10.6 °C/min) and kept at 240 °C for 3.8 min. The determination was made in duplicate. Results were given in area % of total fatty acids. The measurements were performed on the extracts, and the oil used for preparing the mayonnaise and separated oils from mayonnaises collected at day 0 and 28 of the storage experiment.

Primary oxidation products: peroxide value (PV)

Peroxide value of the oil phase separated from mayonnaise ("Lipid phase extraction and oil phase separation") was determined. This was done according to the method by Shantha and Decker [31], based on the formation of an iron–thiocyanate complex. The coloured complex was measured photospectrometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD, USA). The analyses were done in duplicate, and the results are expressed in milli-equivalents peroxides per kg oil (meq O_2/kg).

Analysis of secondary oxidation products: volatiles

Secondary volatile oxidation products were collected using dynamic headspace collection. The collection was carried out using 4 g of mayonnaise (incl. 30 mg internal standard, 4-methyl-1-pentanol) purging with N_2 (flow of 150 mL/min) for 30 min at 60 °C using Tenax GR™ packed tubes. Afterwards, the Tenax tubes were flushed for 20 min with N_2 to remove water. Volatile acids were removed by 0.1 g KOH during the headspace collection, by connection of a S-tube with KOH, as described by Hartvigsen et al. [32]. The trapped volatiles were desorbed using an automatic thermal desorber (ATD-400, Perkin, Norwalk, CT) connected to an Agilent 5890 IIA model (Palo Alto, CA, USA) gas chromatograph equipped with a HP 5972 mass selective detector. Chromatographic separation of volatile compounds was performed on a DB1701 column (30 m × ID 0.25 mm × 0.5 μm film thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min). The temperature program was as follows: 3 min at 35 °C, 3 °C/min from 35–120 °C,

7 °C/min to 120–160 °C, 15 °C/min 160–200 °C and hold for 4 min at 200 °C.

The auto sampler collectors setting details: 9.2 psi, outlet split: 5.0 mL/min, desorption flow: 60 mL/min. The MS unit was set to 70 eV and measured in the mass range between 30 and 350.

Analysis was performed in triplicate, and the results are given in ng/g mayonnaise.

The quantification of the different volatiles was done by the use of a calibration curve prepared from external standards. Solutions with external standards at different concentrations were prepared and added to a fresh mayonnaise sample prepared with neither fish oil nor extracts. Then, the volatiles were collected in the same way as for the samples.

Statistics

Results were expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA) was carried out, and the statistical comparisons among the groups were performed with Bonferroni multiple comparison test. P value <0.05 was considered as statistically significant. All data were analysed with the statistical package program GraphPad prism 4 (GraphPad Software Inc., San Diego, USA).

Principle component analysis (PCA) was carried out using Unscrambler version 9.0 (Camo, Oslo, Norway). Two PCA plots were made, one with iron chelating ability, reducing power, radical scavenging and primary and secondary oxidation products as variables. The second PCA included chelating ability, reducing power, radical scavenging, free amino acids, trace metals, tocopherols and pigments as variables. The PCA model was built on the average of the measured data, all weighting of the values was 1/SD, and full cross-validation was used to validate the model.

Results and discussion

The extraction was mainly meant to extract phenolic compounds from *F. vesiculosus*, including phlorotannins. However, the extraction methods are not specific for phenolic compounds and co-extract a wide range of other bioactive compounds. Three extraction media were used: ethanol, acetone and water. For water extraction, two parts of *F. vesiculosus* were used to investigate the effect on the antioxidative properties of the different biosynthesis of bioactive compounds in the young, growing parts and the old parts of the leaf. Depending on the extraction solvent, the composition of bioactive compounds and their quantity differ.

Extract characterization

Extract composition

In Table 1, the composition of potential bioactive compounds in the extracts is shown. The total phenolic content (TPC) was determined as a quantification of the phlorotannins content. AcE had the highest content of phenolic compounds, 23.2 ± 1.1 GAE/100 g. Also EtE had high TPC (20.4 ± 2.4 GAE/100 g) compared with a relatively low TPC in WoE (6.9 ± 0.3 GAE/100 g) and WyE (12.6 ± 2.1 GAE/100 g).

Our results were in agreement with Wang et al. [13], showing that less polar solvent yields more phenolic compounds. Wang et al. [13] found that when extracting with acetone the phenolic yield was 39.3 PEG/100 g followed by ethanol extract with 35.4 PEG/100 g. Similar to the present study, they also found that extraction with water gave the lowest concentration of phenolic compounds, 26.3 PEG/100 g. These results are higher than what we found,

which can be due to seasonal variation since phenolic content are highly seasonal dependent [33]. In contrast to our finding, Hemmi et al. [11] stated that higher nutrient content in the young leaves causes lower phenolic content, whereas we found that the phenolic content of the young part of the seaweed (WyE) was higher than the old part (WoE).

The protein content was between 1.7 ± 0.0 and 7.2 ± 0.0 % w/w. The protein content in both water extracts was higher than that in EtE and AcE, and WoE had the highest content. The higher content of protein in the water extracts might be due to higher degree of hydrophilic proteins in *F. vesiculosus*, and therefore, these will co-extract with water to a larger extent than with ethanol or acetone.

All four extracts contained tocopherol. This finding is in agreement with the findings of Farvin and Jacobsen [7] who found that brown seaweed, especially the *Fucus* species, contain high amounts of alpha, beta, gamma and delta tocopherols. The most active tocopherol homologue as antioxidant in vitro, delta tocopherol,

Table 1 Extract characteristics

Extracts	Extract composition				Antioxidant properties[a]		
	TPC (g GAE/100 g dry extract)	Protein (%)	Tocopherol α, β, γ, δ (µg/g dry extract)	Iron (µg/g dry extract)	DPPH radical scavenging activity (%)	Metal chelating ability (%)	Reducing power (OD$_{700}$)
Water (old), WoE	6.9 ± 0.3[a]	7.2 ± 0.0	42.2 ± 4.4	61.5 ± 0.4[a]	68.5 ± 0.3[a] x	1.3 ± 0.9[a] x	0.2 ± 0.0[a] x
			5.2 ± 0.5				
			10.4 ± 1.0		75.1 ± 1.5[a]	0.3 ± 3.0[a]	0.2 ± 0.0[a]
			40.2 ± 4.1				
Water (young), WyE	12.6 ± 2.1[b]	4.6 ± 0.2	11.5 ± 4.9	35.2 ± 0.5[b]	98.00 ± 2.1[a] y	14.2 ± 6.1[a] x, y	1.2 ± 0.1[a] y
			2.6 ± 1.1				
			3.00 ± 1.2		97.6 ± 0.7[a]	37.4 ± 6.7[a]	1.5 ± 0.0[a]
			13.00 ± 6.00				
Ethanol, EtE	20.4 ± 2.4[c]	1.7 ± 0.0	2.4 ± 1.9	14.5 ± 0.1[c]	97.6 ± 0.6[a] y	23.0 ± 3.0[a] x, y	1.6 ± 0.0[a] y
			1.7 ± 0.3				
			1.8 ± 0.5		97.8 ± 0.3[a]	24.5 ± 7.0[a]	1.5 ± 0.1[a]
			18.0 ± 4.4				
Acetone, AcE	23.2 ± 1.1[c]	2.3 ± 0.0	4.0 ± 0.3	9.5 ± 1.1[d]	100.7 ± 0.7[a] y	52.3 ± 1.0[a] y	1.6 ± 0.1[a] y
			1.9 ± 0.7				
			2.5 ± 0.8		101.5 ± 0.9[a]	28.9 ± 6.7[a]	1.6 ± 0.1[a]
			12.9 ± 0.6				
Control[b]					90.7 ± 0.2[a] y	94.8 ± 1.6[a] z	1.8 ± 0.1[a] y
					92.6 ± 0.2[a]	95.5 ± 0.1[a]	1.6 ± 0.0[a]

Total phenolic content (TPC), protein content, tocopherol content, iron content and in vitro antioxidant properties (DPPH radical scavenging, metal chelating ability and reducing power)

[a] 1.5 mg extract/mL methanol (upper value) and 2 mg extract/mL methanol (lower value)

[b] Control: The conventional antioxidant used as control in antioxidant assays in concentrations of 1.5 and 2 mg/mL methanol (DPPH radical scavenging activity = BHT, *EDTA* metal chelating ability, reducing power = ascorbic acid): *a, b, c, d*: show significance between extracts in extract composition (TPC and iron) and show significance of increasing antioxidant activity with concentrations for a specific extract in a specific antioxidant assay; *x, y, z*: shows significance between extracts (mean value) in a specific antioxidant assay

was present in higher amounts than other tocopherols, except in WoE, for which the content of alpha tocopherol was higher. The content of alpha tocopherol was higher in the water extracts compared with EtE and AcE. The presence of delta tocopherol in high amounts could influence and enhance the oxidative stability of the extracts.

The iron content of all extracts was significantly different between samples (Table 1). Especially the water extracts, mainly WoE, had a high content of iron. Reduced iron (Fe^{2+}) can act as an initiator of the lipid oxidation and can be considered as a pro-oxidant. The metal content of *F. vesiculosus* can vary a lot between samples harvested at different places and through sessional changes. Variation found in the Øresund region between Denmark and Sweden was from 53 to 151 µg iron/g dry weight *F. vesiculosus* [34]. These findings in seaweed were higher than the iron content in the extracts. Since iron can act as a pro-oxidant in lipid oxidation, a low content was preferable. AcE showed the lowest iron content.

The pigments found belong to the main group of carotenoids and chlorophylls. Whereas AcE showed the lowest pigment content, WoE showed the highest (Table 2). It was found that, especially in the water extracts, high amounts of chlorophyll derivatives were present. The amounts of specific pigments in *F. vesiculosus* was previously reported to be 0.2 µg/mg dry weight (DW) for beta carotene, 1 µg/mg DW fucoxanthin and 5 µg/mg DW chlorophyll a [35]. Our findings differed from these values. This can be due to natural variations and the extraction process. It is possible that during the extraction process, pigments were destroyed or oxidized. Notable is that Nygard and Ekelund [35] has found approximately 2.5 times more chlorophylls than carotenoids in *F. vesiculosus*; however, we found the opposite in our extracts. This can be due to that chlorophylls in our extracts were unstable and therefore little chlorophyll was found in EtE and AcE and a relatively high amount of chlorophyll derivate (DV) in the water extracts. Fucoxanthin is known as a characteristic pigment in brown algae, and its radical scavenging ability is reported in the literature [36].

Chlorophyll can work as sensitizer in photooxidation [37]. Therefore, high content of potential photooxidizers can be a problem in application of these extracts in foods.

Another issue is the breakdown of chlorophylls to chlorophyll derivates during extraction. We do not know which kinds of derivates were formed and the role they play in oxidation in food. The carotenoids present in the extracts were not converted to derivates during the extraction process, so it can be assumed that carotenoids have an antioxidant effect in the extracts [38].

Table 2 Pigment content of the four extracts

Pigment (µg/mg)	Extracts			
	EtE	AcE	WoE	WyE
Chlorophyll C2	1.5 ± 0.2	0.6 ± 0.1	1.1 ± 0.1	0.6 ± 0.0
Chlorophyllide a	0.5 ± 0.0	ND	ND	ND
DV chlorophyll a	ND	ND	2.7 ± 0.1	0.8 ± 1.1
Chlorophyll a	ND	0.2 ± 0.0	0.1 ± 0.2	ND
19-But-fucoxanthin	4.9 ± 0.1	0.4 ± 0.1	2.9 ± 0.1	1.3 ± 0.1
Fucoxanthin	0.9 ± 0.4	ND	ND	0.6 ± 0.8
Prasinoxanthin	ND	ND	1.4 ± 0.0	0.3 ± 0.1
19-Hex-fucoxanthin	0.7 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
Astaxanthin	0.9 ± 0.2	ND	0.5 ± 0.1	0.2 ± 0.0
Dinoxanthin	ND	ND	0.4 ± 0.0	0.2 ± 0.0
Zeaxanthin	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.3	ND
Lutein	ND	ND	ND	0.4 ± 0.0
Carotene *a + b*	ND	ND	2.4 ± 0.0	0.6 ± 0.8
Total	9.6	1.6	13.6	5.2

EtE ethanol extract, *AcE* acetone extract, *WoE* aqueous extract from old parts of the leaf, *WyE* aqueous extract from young parts of the leaf

ND not detectable

Antioxidant potential of *F. vesiculosus* extracts

Since an efficient antioxidant possess both radical scavenging ability and chelating activity, multifunctional antioxidants are more efficient in complex food emulsions such as mayonnaise [21]. Therefore, the antioxidant properties of the seaweed extracts were determined by in vitro antioxidant assays to evaluate their potential as novel natural antioxidants in foods. The results are shown in Table 1.

All extracts, except the WoE, had the same radical scavenging ability as BHT, close to 100 %. The radical scavenging properties of WoE were concentration dependent ($P < 0.0001$) and increased with increasing concentration (68.5 ± 0.4–75.1 ± 1.5 %). These findings are in agreement with results from Wang et al. [12] who found that acetone extract was the most efficient radical scavenger, whereas water extracts were less efficient. The fact that concentration dependence was only observed for WoE may be because the radical scavenging ability already had reached its maximum at the lowest concentration for the other extracts and BHT. High radical scavenging ability can be related to a high TPC in *F. vesiculosus* extracts, as it has been stated by Wang et al. [12]. Only WoE extract confirms the TPC dependence, since this extract had the lowest TPC and also the lowest radical scavenging ability. It can be assumed that the concentration of the extracts used in the assays was too high to see both TPC and concentration dependence. However, it could not be excluded that other compounds present acted as radical scavengers.
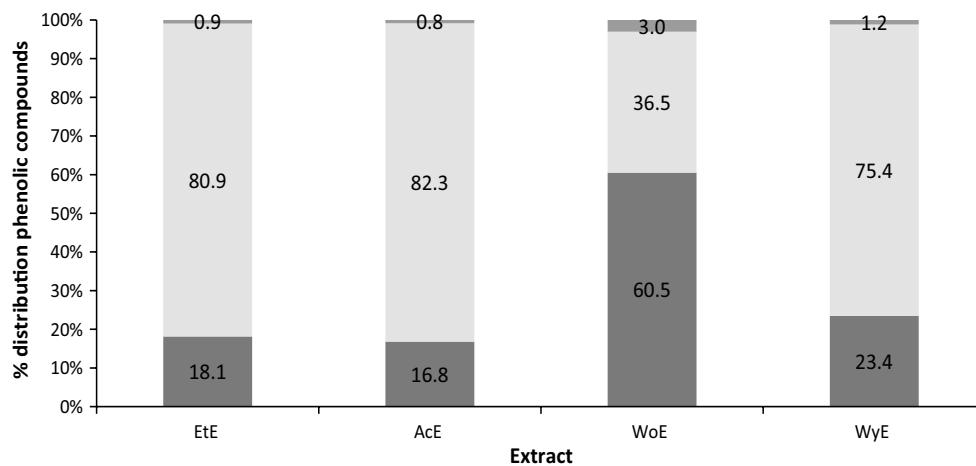
Fig. 1 Per cent phenolic compounds (%) in octanol (*grey*), water (*light grey*) and interface (*dark grey*) of a partitioning study. $P_{o/w}$ was −1.3 showing that the extracts were hydrophilic. *EtE* ethanol extract, *AcE* acetone extract, *WoE* aqueous extract from old parts of the leaf, *WyE* aqueous extract from young parts of the leaf

For example, sulphated polysaccharides are present in *F. vesiculosus* and are assumed to act as radical scavengers [39].

All extracts possessed metal chelating ability. It was found that all extracts exhibited lower metal chelating ability than EDTA. The WoE extract showed a significantly lower activity than the other extracts at all concentrations. No correlation between increasing concentrations and increasing metal chelating ability was found. Wang et al. [12] found that the iron chelating activity of *F. vesiculosus* water extract was almost 100 % and approximately 60 % in acetone extract at a concentration of 5 mg/mL. Our findings showed lower values indicating that the iron chelating activity could be concentration dependent since the highest concentration used in our study was only 2 mg/mL. However, a concentration-dependent pattern was not found in our study. According to Jacobsen et al. [20], the main cause of oxidation initiation in mayonnaise is the presence of metals, especially iron. Therefore, metal chelating activity is crucial in o/w emulsion systems like mayonnaise.

EtE and AcE exhibited the highest reducing power, whereas the reducing power of WoE was lowest. A high difference between reducing power of WoE and WyE extracts was found. Significant difference in reducing power of different concentration was only found in the WyE extract ($P < 0.001$), as the reducing power increased with increasing concentration. No concentration dependence like this was found for the other extract. EtE and AcE at 1500 µg/mL were already reaching the maximum reducing power. Nevertheless, it cannot be ruled out that there could be concentration dependence of EtE and AcE at lower concentrations.

Partitioning of phenolic compounds

Apart from the radical scavenging and metal chelating activities and reducing power, the efficacy of antioxidants is also highly influenced by their location in o/w emulsions. The octanol/water partitioning coefficient (logP) for the TPC was determined for all four extracts. This was done by determining TPC in both the octanol and water phase. TPC of the interfaces was then calculated based on TPC of the whole extract, determined in "Extract composition". The distribution (%) of phenolic compounds in the water, octanol or interface is shown in Fig. 1.

logP was found to be −1.30 ± 0.00 for all extracts which indicates that the phenolic compounds were hydrophilic and mainly located in the water phase.

The highest percentage of phenolic compounds was found in the interface, and the interface of EtE had the highest content (16.5 GAE/100 g extract) followed by AcE (19.1 GAE/100 g extract), WoE (2.5 GAE/100 g extract) and WyE (9.5 g GAE/100 g extract). This was probably due to phenolic–protein interactions with proteins present in the extracts as described by Porter [40]. It can be hypothesized that the presence of phenolic compounds in the interface increases the antioxidant properties in mayonnaise because the phlorotannins cover the oil droplets and protect them against radicals and oxidation initiators from the water phase.

Oxidative stability in fish-oil-enriched mayonnaise with seaweed extracts

To investigate how well the extract worked as antioxidants in foods, the oxidative stability of the fish-oil-enriched mayonnaise added the extracts was evaluated. This was
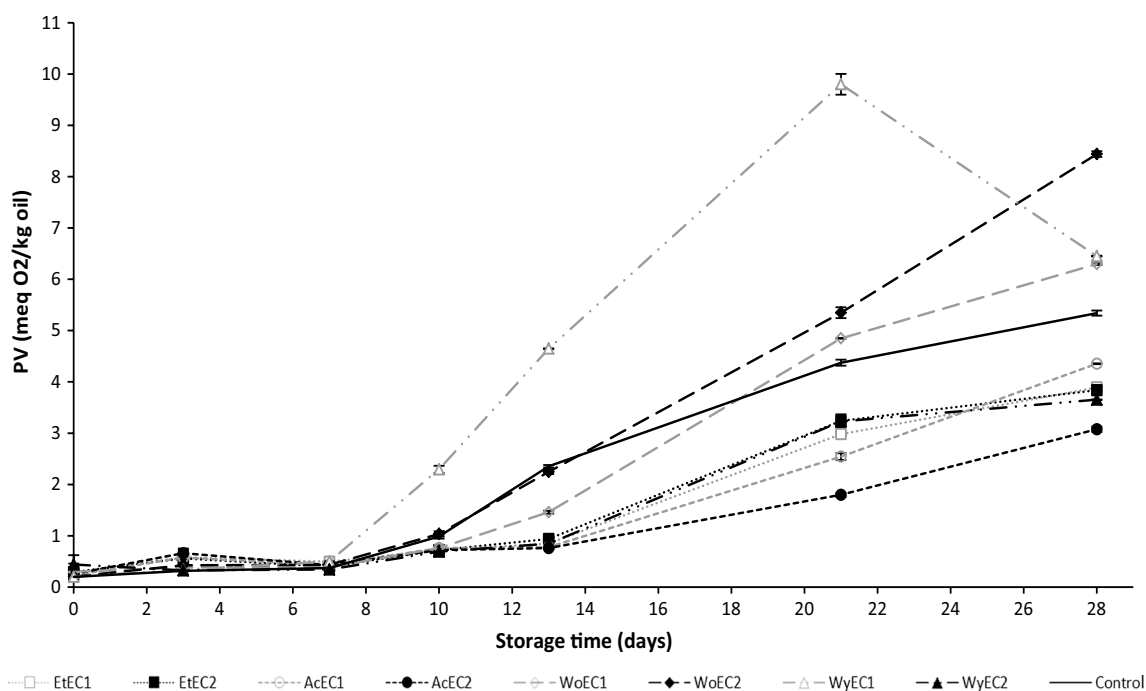
Fig. 2 Development in peroxide value (PV) in fish-oil-enriched mayonnaise with or without seaweed extract during 28 days of dark storage at 20 °C. *EtE* ethanol extract, *AcE* acetone extract, *WoE* aqueous extract from old parts of the leaf, WyE = aqueous extract from young parts of the leaf. C1 = 1.5 g/kg mayonnaise, C2 = 2 g/kg mayonnaise

done by determining changes in tocopherol contents, the development in primary oxidation by peroxide value and volatile secondary oxidation product during storage. For the secondary oxidation products, nine volatile compounds (2-ethylfuran, pentanal, 1-penten-3-ol, 2-pentenal, 2-hexenal, nonanal, hexanal, 1-penten-3-one and 2,4-heptadienal) were detected and quantified by dynamic headspace GC-MS.

Formation of primary oxidation products

In Fig. 2, the formation of hydroperoxides during storage is shown. A lag phase was found until day 7 for WyEC1, WoEC1, WoEC2 and the Control for low (C1) and high concentration (C2). For the other samples a lag phase up to day 13 was observed. Similar patterns in primary oxidation production were found by Hermund et al. [15] for ethyl acetate fractions and water extracts of *F. vesiculosus*. AcE and EtE had the lowest formation of hydroperoxides at the end of storage compared to the water extracts, which indicates an antioxidative effect in mayonnaise. The mayonnaises with the water extracts (except WyEC2) contained more hydroperoxide than the Control mayonnaise from day 13 on, which indicates a pro-oxidative effect. In this study, WyEC1 showed pro-oxidative properties in mayonnaise, whereas WyEC2 showed antioxidant properties. The difference between WyEC1 and WyEC2 could be explained by a concentration-dependent pro-oxidative effect in low

concentration, whereas in higher concentration the antioxidant properties overrule. Furthermore, mayonnaise added WyEC1 extract was found to have the highest content of hydroperoxides until day 20. Then, the amount decreased.

Formation of secondary oxidation products

Formation of secondary oxidation products, including those related to oxidation of fish oil, was determined. Whereas 2-ethylfuran, pentanal, 1-penten-3-ol, 2-pentenal, 2-hexenal, nonanal, hexanal, 1-penten-3-one and 2,4-heptadienal are all correlated to oxidation of fish oil, pentanal, hexanal and nonanal are correlated to both the oxidation of fish oil and rapeseed oil in fish-oil-enriched mayonnaise [32].

In general, the development of volatile oxidation products during storage was found to follow the same pattern. In Fig. 3, the formation of 1-penten-3-ol and 2-hexenal are shown, showing this pattern. A lag phase in production of 1-penten-3-ol was found within the first 7 days for WyEC1 and the Control, whereas AcEC2 had a lag phase up to day 21 of storage. For 2-hexenal, lag phases were not as clear as for 1-penten-3-ol since the concentration of 2-hexenal increased slightly from day 0. However, the increase pattern of 2-hexenal was still comparable with the increase in 1-penten-3-ol.

The formation of pentanal (data not shown) had no lag phase since the concentration increased from day 0 and throughout the storage. 2,4-Heptadienal (data not shown)
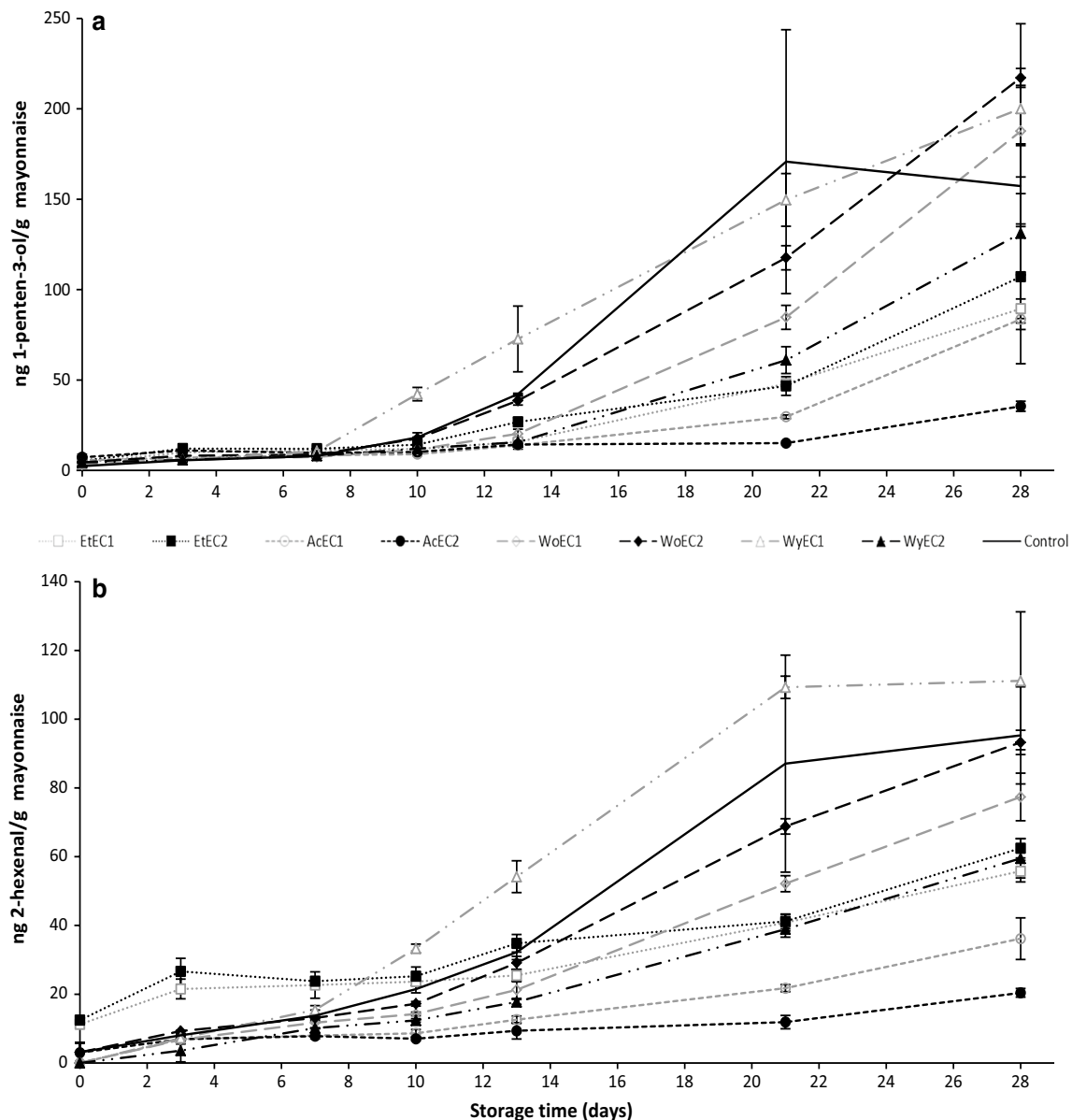
Fig. 3 Development of 1-penten-3-ol (**a**) and 2-hexenal (**b**) in fish-oil-enriched mayonnaise with or without seaweed extract during the 28 storage days at 20 °C. *EtE* ethanol extract, *AcE* acetone extract, WoE = aqueous extract from old parts of the leaf, WyE = aqueous extract from young parts of the leaf. C1 = 1.5 g/kg mayonnaise, C2 = 2 g/kg mayonnaise

showed an initial amount of about 1000 ng/g mayonnaise indicating a natural presence of this compound in fresh mayonnaise.

The concentration of hexanal, nonanal and 1-penten-3-one did not change during storage and were therefore not linked to oxidation in the mayonnaise in this case (data not shown). Regarding the antioxidant activity, the same findings were seen as in the primary oxidation products. The AcE and EtE were more efficient in inhibiting lipid oxidation than the water extracts. Also Hermund et al. [15] found that water extract of *F. vesiculosus* was less efficient in preventing oxidation in mayonnaise compared to an ethyl acetate extract.

Tocopherol changes during storage

Tocopherol changes during storage were determined to investigate whether there was a reduction in some of the tocopherol homologues during storage due to consumption of tocopherols to prevent lipid oxidation. However, no reduction in tocopherol homologues was found between day 0 and 28 of storage and data are therefore not shown. This indicates that the main antioxidant activity is due to the extracts and not due to the presence of tocopherols. Nevertheless, it cannot be ruled out that the tocopherols influence the oxidative stability and the extracts might have a regenerating effect on tocopherols.
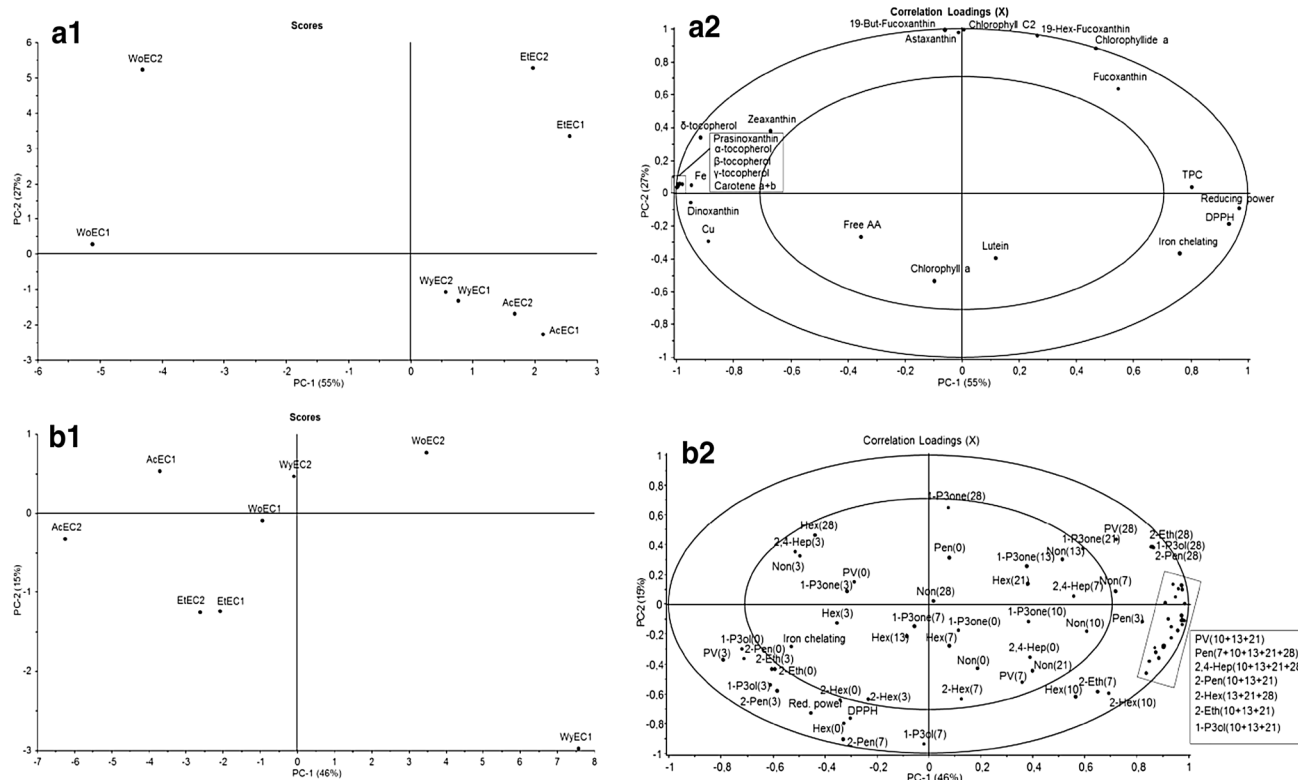
Fig. 4 Principal component analysis (PCA). **a** is the PCA of the extract composition parameters and the antioxidant properties, and **b** is the PCA of antioxidant properties (iron chelating, *red* power, DPPH), peroxide value (PV) and all secondary oxidation products (sampling days are shown in brackets). The score plots (1) show the scores/the extracts in the two concentrations (C1 and C2) and the loading plot (2) contains all the variables. *2-Eth* 2-ethylfuran, *1-P3one* 1-penten-3-one, *Pen* pentanal, *1-P3ol* 1-penten-3-ol, *2-Pen* 2-pentenal, *Hex* hexanal, *2-Hex* 2-hexenal, *2,4-Hep* 2,4-heptadienal, *Non* nonanal. *EtE* ethanol extract, *AcE* acetone extract, *WoE* aqueous extract from old parts of the leaf, *WyE* aqueous extract from young parts of the leaf. C1 = 1.5 g/kg mayonnaise, C2 = 2 g/kg mayonnaise

Principal component analysis (PCA)

Two PCAs (Fig. 4) were carried out to gain an overview of the similarities and differences among the four extracts with respect to their effects in mayonnaise, in vitro antioxidant properties and bioactive compounds. A PCA was made to investigate the relationship between the extracts and their composition and in vitro antioxidant properties. The first two principal components in this PCA explained 55 and 27 % of the total variance in the data set, respectively (Fig. 4a). In particular, AcE and EtE which were correlated to a higher concentration of TPC showed a higher correlation with the in vitro antioxidant properties, indicating that TPC influences the antioxidant properties of AcE and EtE. Whereas AcE and EtE were found to be correlated with TPC, WoE was correlated more with tocopherols and trace metals. Regarding pigments, the different pigments are spread all over the PCA loading plot, and this makes it difficult to correlate them with the antioxidative effects. Nevertheless, it cannot be ruled out that, e.g., fucoxanthin contributed to the antioxidant efficiency of the extracts, due

to its higher degree of correlation with the in vitro antioxidant properties.

Another PCA was done to investigate the correlation between the extracts and the oxidation products during storage. The first two principal components explained 46 and 15 % of the total variance in the data set, respectively (Fig. 4b). This PCA showed a negative correlation of all oxidation products with AcE, whereas WyEC2, WoEC1 and EtE in both concentrations were correlated with oxidation products in the initial phase of the storage experiment. The EtE was also correlated with iron chelating ability, reducing power and radical scavenging ability (DPPH). Only WoEC2 showed a higher correlation with oxidation products of later sampling days during storage. A trend that oxidation products in the beginning of the storage are located to the left and later sampling days to the right was found. As found in the antioxidant evaluation of the extracts in fish-oil-enriched mayonnaise, AcE and EtE were the most efficient antioxidants, whereas the water extracts were less efficient, which can be due to the higher trace metal content of the water extracts. This was again confirmed from

the PCA on all the variables measured during the storage experiment.

Conclusion

Results showed a higher efficacy of AcE and EtE compared to the two water extracts in fish-oil-enriched mayonnaise irrespective of the water extracts from the young or old parts of the leaf. The highest concentration of extracts (2 g/kg mayonnaise) in fish-oil-enriched-mayonnaise resulted in enhanced oxidative stability. Whereas the highest concentration of WyE in mayonnaise worked as an antioxidant, the lowest concentration had pro-oxidant activity. Moreover, the efficacy of the extracts was related to high TPC and high radical scavenging activity and probably to the location of phenolic compounds at the interface. The pro-oxidative properties of some water extracts might be due to a higher content of lipid oxidation inducing trace metals. Based on this, especially the AcE and EtE from *F. vesiculosus* showed promising results as replacement of synthetic antioxidants in foods.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with Ethics Requirements This article does not contain any studies with human or animal subjects.

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PAPER III

Antioxidant efficacy of novel brown seaweed based antioxidants on the oxidative stability and microstructure of 5% fish-oil-enriched granola bars

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The effect of novel brown seaweed based antioxidants on the oxidative stability and microstructure of 5% fish-oil-enriched granola bars

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1 **The effect of novel brown seaweed based antioxidants on the oxidative stability and microstructure**

2 **of 5% fish-oil-enriched granola bars**

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16

18

19 **Abbreviations**

20 AE - Samples added acetone extract, CLSM - confocal light scanning microscopy, DHA - docosahexaenoic

21 acid, EE - Samples added ethanol extract, EPA - eicosapentaenoic acid, ESEM - environmental scanning

22 electron microscopy, FO - Fish oil, LC PUFA - Long chain poly unsaturated fatty acid, PV - Peroxide value,

23 RDA% - The relative decrease in area %, SEM - traditional scanning electron microscopy, WE - Samples

24 added water extract

25

26 **Abstract**

27 The aims of this study were to; 1) investigate the ability of Icelandic brown algae *Fucus vesiculosus* extracts

28 to inhibit lipid oxidation in granola bars fortified with fish oil-in-water emulsion; 2) investigate whether addition

29 of the seaweed extracts affected the physical microstructure of the oil droplets in granola bars.

30 The oxidative stability of the bars at 20°C was evaluated over a period of 10 weeks by measuring the

31 development of peroxides and volatile compounds using dynamic headspace gas chromatography mass

32 spectrometry (DHS GC-MS). The physical microstructure was determined using microscopy.

33 All extracts - except water extract in low concentration - reduced lipid oxidation during 10 weeks of storage

34 when added in a concentration of 0.5 or 1 g extract/100 g emulsion. Ethanol and acetone (lowest

35 concentration) extracts were found to be most efficient as antioxidants in the bars. The antioxidant efficacy of

36 these two extracts was among other related to an improved incorporation of the fish oil-in-water emulsions in

37 the bars, high total phenolic content, high radical scavenging activity together with high interfacial affinity of

38 phenolic compounds and probably regeneration of tocopherol.

39 *Practical applications*: The work showed the application potential of *Fucus vesiculosus* extracts as a natural

40 antioxidant in low-moisture foods such as granola bars. These findings implied that the multi-functional

41 nature of these extracts provides not only oxidative stability of the food but also a physical stability.

42

1.   Introduction

Lipid oxidation in foods has been counted as a major problem due to its detrimental effects on the quality of food including formation of a wide range of unpleasant odours and rancid taste. During the last couple of decades, there has been an increasing demand for functional foods enriched with n-3 long chain polyunsaturated acid (LC PUFA) of marine origin, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) due to their health benefits [1]. However, n-3 LC PUFAs are highly susceptible to oxidation [2] and enrichment of food products with fish oil (FO) decreases the oxidative stability [3]. Therefore, it is necessary to control oxidation of n-3 LC PUFAs to prevent deteriorative quality changes in functional foods containing FO.

Monomolecular antioxidants such as butylated hydroxytolulene (BHT) and ethylenediaminetetraacetic acid (EDTA), tocopherol and ascorbic acid are in many cases not able to prevent oxidative flavour deterioration in FO enriched foods [3, 4]. Hence, there are emerging interests in finding multi-functional antioxidants preferably of natural origin.

Brown algae, *Fucus vesiculosus*, are rich in the bioactive polyphenolic secondary metabolites, *phlorotannins* [5, 6, 7, 8]. Studies of these compounds extracted from *F. vesiculosus* have shown that they possess both radical scavenging activity and metal chelating ability *in vitro*, which makes them well suited as antioxidants in foods. Furthermore, *F. vesiculosus* contains a wide range of other substances like pigments, such as carotenoids, tocopherol, sulphated polysaccharides, peptides, amino acids, metals, and polyphenols, which all can exhibit some degree of antioxidant, prooxidant or synergistic activity [9]. Together with the phlorotannins these substances possibly makes up for multiple function of extracts from seaweed.

Previous studies of extracts from *F. vesiculosus* found excellent antioxidant efficacy of these in food emulsion systems, such as milk and mayonnaise [10, 11]. However, it can be difficult to extrapolate this antioxidant efficacy from one food system to another, the application of these extracts need to be evaluated in other food, such as low-moisture foods.

In a study by Nielsen and Jacobsen [12] the oxidative stability of energy bars enriched either with neat FO, fish-oil-emulsion or micro-encapsulated FO powder was studied to investigate which delivery system was best suited for the enrichment of the energy bars. It was found that the bars enriched with emulsions were more stable to lipid oxidation than the bars enriched with neat FO. Furthermore, in contrast to other studies EDTA had pro-oxidative activity in energy bars indicating that metal chelating ability has a minor importance

72 in this type of low-moisture food [3]. In a follow up study, selected antioxidants with hydrophilic, amphiphilic

73 and lipophilic properties were added to the energy bars enriched with fish-oil-emulsion. It was found that only

74 the lipophilic antioxidant tocopherol reduced lipid oxidation when it was added in concentrations above 440

75 µg/g FO. Other antioxidants (caffeic acid and ascorbyl palmitate) showed pro-oxidative effects when tested

76 in concentrations from 75 to 300 µg/g FO [13]. However, more knowledge regarding the incorporation of the

77 fish-oil-emulsions in energy bars and their physical microstructure, which may be affected by the antioxidant

78 addition, is needed to understand how the oxidative stability depends on the incorporation of the emulsions

79 into the food matrix.

80 The aims of this study were, firstly to investigate the ability of Icelandic *F. vesiculosus* extracts to inhibit lipid

81 oxidation in granola bars fortified with a 70% FO-in-water emulsion and secondly to investigate whether

82 addition of the seaweed extracts affected the physical microstructure of the oil droplets in granola bars. Fish-

83 oil-emulsions were produced with or without seaweed extract and the emulsions were added to granola bars.

84 The oxidative stability of the bars at 20°C was evaluated over a period of 10 weeks by measuring the

85 development of peroxides and volatile compounds using dynamic headspace gas chromatography mass

86 spectrometry (DHS GC-MS) together sensory evaluation performed by an expert sensory panel. The

87 incorporation of the fish-oil-emulsions and the physical microstructure of the granola bars were determined

88 using three microscopy methods; confocal light scanning microscopy (CLSM), traditional scanning electron

89 microscopy (SEM) and environmental scanning electron microscopy (ESEM).

90

91 **2.   Material and methods**

92 *2.1.   Materials*

93 The ingredients included soft brown sugar and syrup (Dansukker, Nordic Sugar, Denmark), honey (Jakobsen

94 A/S, Denmark), wheat flour (Flechtorfer Mühle GmbH, Germany), rolled oats (Lantmännen Cerealia A/S,

95 DK), Kellogg's Rice Crispies (Nordisk Kelloggs ApS, Denmark), raisins (K.F.C. Gida A/S, Turkey), apricots

96 (Elmas Dis Ticaret A/S, Turkey), and fig/cocoa spread: nut/fig mix (Castus A/S, Denmark), hazelnuts and

97 cocoa powder without added sugar (Coop, Denmark), and water. All ingredients were purchased from the

98 local supermarket. Sodium caseinate was provided by Arla (Arla Foods Ingredients amba, Viby J., Denmark),

99 maltodextrin (Viking nutrition, NK Import, Denmark), and FO (maritex 43-01) by Maritex (Maritex, Sortland,

100 Norway). The FO, used in the emulsions for the bars, had a peroxide value (PV) of 0.8 meq peroxides/kg oil

101 and a tocopherol content of 418 µg α-tocopherol/g oil, 0 µg β-tocopherol/g oil, 192 µg γ-tocopherol/g oil and

102 62 µg δ-tocopherol/g oil. The fatty acid composition (only the content ≥ 0.5%) was as follows: 14:0 (3.6 %);

103 16:0 (10.3 %); 16:1 (n-7) (8.9 %); 16:2 (n-4) (0.5 %); 18:0 (2.2 %); 18:1 (n-9) (15.8 %); 18:1 (n-7) (4.5 %);

104 18:2 (n-6) (1.9 %); 18:3 (n-3) (0.9 %); 18:4 (n-3) (2.4 %); 20:1 (n-11+n-9) (13.8 %); 20:1 (n-7) (0.6 %); 20:4

105 (n-3) (0.7 %); 20:5 (n-3) (EPA) (8.7 %); 22:1 (n-9) (6.5 %); 22:1 (n-11) (0.8 %) and 22:6 (n-3) (DHA) (10.9

106 %). All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Internal

107 standards were purchased from Sigma Aldrich (Steinheim, Germany).

108

109 *2.2.   Extract production from Icelandic Fucus vesiculosus*

110 Extraction was performed by Matís in Iceland according to [6, 8]. The *F. vesiculosus* was collected in the

111 Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in 2011. At the collecting spot the

112 seaweed was washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of

113 the samples and transported to the laboratory. The samples were carefully rinsed with tap water, wiped with

114 paper towel. They were then freeze-dried, pulverised into powder and stored at -80 °C prior to extraction.

115 The water, acetone and ethanol extracts were produced as follows: Five grams of the algal powder was

116 mixed with either, 100 mL of distilled water, 70 % aqueous acetone (v/v) or 80 % ethanol (v/v), and

117 incubated in a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 124 g at

118 room temperature. The mixture was centrifuged at 2168 g for 10 min at 4 °C and filtered with Whatman no. 4

119 filter paper. Each extraction was conducted in duplicate. The water (WE), acetone (AE) and ethanol (EE)

120 extract were stored at -80 °C until used for application.

121

122 *2.3.   Production of 70% FO-in-water emulsions*

123 FO-in-water emulsions were prepared according to Horn et al. [13]. The 70% (w⁄w) FO-in-water emulsions

124 were prepared at room temperature by slowly adding FO to a 10% (w/v) sodium caseinate (Na-cas) in water

125 solution while vigorously mixing with an ultra-turrax at approximately 20,000 rpm until all FO was added. The

126 pH of the emulsions was approximately 7. The seaweed extracts were dissolved in 10% Na-cas solution

127 (w/v) having 0.5 or 1 g extract in 100 g emulsion. These two concentrations are indicated in the sample

128 codes as 1 or 2, respectively.

129

2.4. Droplet size distribution

The size of the oil droplets in the FO-in-water emulsions was determined using a Mastersizer2000 (Malvern Instruments, Worcestershire, UK). The droplet size was measured the day after production by dissolving FO-in-water emulsion in SDS buffer (1 g emulsion and 5 g SDS buffer) (10 mM NaH_2PO_4, 5 mM SDS, pH 7) by sonication for 15 min. The Mastersizer2000 was set up with a refractive index of material of 1.4694 (oil) and of dispersant of 1.3333 (water). The samples were added drop wise directly into the recirculating water until an obscuration between 12-17% was reached at 3000 rpm. The droplet size was measured three times. The average of the measurements was taken for further calculation. Results were expressed in $D[3,2] = \sum d^3/d^2$ [14].

2.5. Production of 5% FO-enriched granola bars and sampling

The production of granola bars was carried out according to Horn et al. [13] with some alterations. The ingredients were weighted according to Table 1. Firstly, the nut/fig/cocoa spread was prepared. Hazelnuts were toasted and blended (Kenwood kitchen blender) together with nut/fig mix, cocoa powder and water to a smooth paste. All dry ingredients; wheat flour, rolled oats, maltodextrin, rice crispies, raisins and apricots (chopped finely by hand) were mixed. Separately, all wet ingredients; soft brown sugar, syrup, honey, nut/fig/cocoa spread and the FO emulsion were mixed. Subsequently, the wet mixture was poured onto the dry ingredients. Final mixing was carried out on a Viking mixer (20PS, Seidefors Agenturer AB, Bandhagen, Sweden) for 1 min at speed 60. The dough was baked in tin foil trays (500 g volume) with 350 g in each tray (equals one batch) for 15 min at 175°C. After cooling at room temperature, each batch was cut into portions of 35 g for both chemical and sensory analysis. The granola bars was packed in sealed plastic bags at atmospheric conditions and stored at room temperatures (Average 21 °C) in the dark for up to 10 weeks. Dry matter (%) of bars was determined on week 0 samples and is given in Table 2.

2.6. Iron and Cupper content

The samples were destructed prior to metal analysis: 0.5 g sample were mixed with 5 mL HNO_3 (68%), 3 mL H_2O_2 (30%) and 0.5 mL HCl (37%). Two reference samples with fish protein certified reference material for trace metals (DORM-3) (National Research Council, Ottawa, ON, Canada) were also prepared. Destruction was carried out in a destruction oven (Multiwave 3000, Anton Paar, Graz, Austria), in which the samples

159 were heated for 10 min at 1400 W. After destruction, the samples were kept at room temperature for 20 min

160 before being transferred into 10 mL volumetric flasks and filled with water. Prior to analysis, samples were

161 diluted to reach acidity below 2% HNO_3 and 1% HCl. Analysis was performed using Inductively Coupled

162 Plasma Mass Spectrometry (ICPMS) (ICPMS model 7500ce, Agilent Technologies, Wilmington, DE, USA)

163 equipped with a Scott-type spray-chamber and a concentric nebulizer with a CETAC autosampler (ASX-500,

164 CETAC Technologies, Omaha, NE, USA). The analysis was done on the isotopes 57Fe and 63Cu with the

165 ICPMS in collision-reaction-cell (CRC) mode using helium as cell-gas for interference reduction. The

166 calibration was done using external calibration with standard solutions in the concentration range from 0-200

167 µg/L for both elements. The calibration standards were prepared by dilution of certified single-element stock

168 solutions (SCP Science, Courtaboeuf, France) of 1000 mg/L. The analysis was performed in duplicates.

169

170 *2.7. Extraction of lipids*

171 Lipids were extracted with a homogeneous mixture of chloroform, methanol, and water (2:2:1.8), following

172 the method of Bligh and Dyer [15]. The method was modified to use a smaller volume of solvents as

173 described by Iverson et al. [16], but the original ratio between chloroform, methanol, and water was

174 maintained. Five grams of sample was used for lipid extraction. The lipid extracts were used for the

175 subsequent determination of fatty acid composition and lipid content. The lipid content was determined

176 gravimetric after evaporation of chloroform. Duplicate analyses of each sample were performed.

177

178 *2.8. Fatty acid composition (fatty acid methyl esters, FAME)*

179 The fatty acid composition of the oil phases was determined after fatty acid methylation and analysis by GC-

180 FID. 100 µL toluene, 200 µL heptane with 0.01 % (v/v) BHT and 100 µL internal standard (C23:0) (2 % w/v)

181 were added to an amount of lipid extract corresponding to 30-60 mg lipid. One mL of BF3 in methanol was

182 added to the lipid extract mixture and the lipids were methylated in a one-step procedure using a microwave

183 oven (Multiwave3000 SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave

184 were 5 min at 500 Watt followed by 10 min cooling. The fatty acid methyl esters (FAMEs) were washed with

185 1 mL saturated NaCl and 0.7 mL heptane with 0.01 % (v/v) BHT. The heptane phase was transferred to a

186 GC vial and FAMEs were analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according

187 to AOCS [17]. For separation DB127-7012 column (10 m x ID 0.1 mm x 0.1 µm film thickness, Agilent

188 Technologies, Palo Alto, CA, USA) was used. Injection volume was 0.2 µL in split mode (1:50). The initial

189 temperature of the GC-oven was 160°C. The temperature was set to increase gradually being as

190 follows: 160 -200 °C (10.6 °C/ min), 200 °C kept for 0.3 min, 200 - 220 °C (10.6 °C/ min), 220 °C kept for 1

191 min, 220 - 240 °C (10.6 °C/ min) and kept at 240 °C for 3.8 min. The determination was made in

192 duplicates. Results were given in area %. The relative decrease in area % (*RDA%*) was calculated for EPA,

193 DHA, 18:4 (n-3) and the total PUFAs; ((Area%(week0)-Area%(week10))/Area%(week0))*100%.

194

195 *2.9. Tocopherol content*

196 The lipid extracts were (after evaporation under nitrogen) dissolved in heptane and analysed by high-

197 performance liquid chromatograph (HPLC) (Agilent 1100 Series, Agilent Technology, Palo Alto, CA, USA)

198 according to AOCS [17] to quantify the content of α-, β-, γ- and δ-tocopherol in the samples. These

199 tocopherol homologues were separated using a silica column (Waters (Dublin, Ireland), 150 mm, 4.6 mm, 3

200 µm silica film). A stock solution containing known amounts of α-, β-, γ- and δ-tocopherol was prepared and

201 used for quantification. Samples were analysed at week 0, 2, 4, 6, 8 and 10. The analysis was performed in

202 duplicates and results were reported as µg tocopherol/g granola bar.

203

204 *2.10. Primary oxidation products, PV*

205 PVs of the lipid extracts were determined according to the method by Shantha and Decker [18], based on

206 the formation of an iron-thiocyanate complex. The coloured complex was measured spectrophotometrically

207 at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia, MD). Samples were analysed at

208 each sampling point from week 0 to 10. The analysis was performed in duplicate and the results are

209 expressed in milli-equivalents peroxides/kg oil (meq O_2/kg oil).

210

211 *2.11. Secondary oxidation products, volatiles*

212 The collection of the volatile compounds was carried out using approximately 4 g of crushed granola bar

213 sample (including addition of 30 mg internal standard, 4-methyl-1-pentanol) mixed with 25 mL distilled water.

214 The volatile secondary oxidation products were collected at 45ºC by purging with nitrogen for 30 min at 340

215 mL/min. Hereafter the tubes were flushed with nitrogen (flow of 50 mL/min) for 20 min to remove water from

216 the Tenax GR™ tube. The trapped volatiles were desorbed from the Tenax tubes by heat (200°C) using an

217　automatic thermal desorber (ATD-400, Perkin Elmer, Norwalk, CT), cryofocused on a cold trap (-30°C),

218　released again (220°C), and transferred to a GC Agilent 5890 IIA model (Palo Alto, CA, USA) equipped with

219　a HP 5972 mass selective detector. Chromatographic separation of volatile compounds was performed on a

220　DB1701 column (30 m x ID 0.25 mm x 0.5 µm film thickness, J&W Scientific, Folsom, CA) using helium gas

221　flow (1.3 mL/min). Initial oven temperature was 45°C, rising by 2.0°C /min to 80°C followed by an increase of

222　3.0°C/min to 150°C and finally increased by 12.0°C/min to 240 °C. The individual compounds were analysed

223　by mass spectrometry (MS) (HP 5972 mass-selective detector; Hewlett Packard), identified by MS-library

224　searches and addition of the internal standard, and quantification through calibration curve made by adding

225　the standards directly on the Tenax tubes. For the quantification, a stock solution of 16 volatiles was

226　prepared and a calibration curve was conducted in a range from 0-2 mg/g. The analysis was carried out in

227　triplicates and results are given as concentration (ng/g granola bar).

228

229　*2.12.　Sensory evaluation*

230　A preliminary sensory evaluation was carried out by an expert panel (minimum three persons), rating the

231　fish-oil-enriched granola bars from 0-10, where 0 was "none" (attributes not detectable) and 10 was "very

232　much" (high intensity of the attributes). The evaluation was made based on six sensory parameters (fishy

233　odour and taste, rancid odour and taste, or sweet/brown sugar odour and taste) according to Horn et al. [13].

234　The granola bars were given a code (four ciphers) and were served in a randomised order. Prior to the

235　evaluation the granola bars where cut into three or four equally sized portions and served at room

236　temperature on a tin foil tray. The expert panel evaluated the samples and decided on a common rating of

237　each sample (no statistics applied). Between each tasting the panel cleansed their mouth with water.

238

239　*2.13.　Microscopy*

240　SEM, ESEM and CLSM where performed on week 0 samples added the seaweed extracts in the

241　concentrations of 1 g extract/100 g emulsion. The microscopic methods were performed as follow:

242　*SEM and ESEM.* In order to create fresh fracture surfaces in the chewy and pliable granola bars without

243　creating structural distortion from cutting or pulling, pieces of granola bars were submerged in liquid nitrogen

244　until frozen and then fractured. Fresh fractured samples were quickly mounted on SEM stubs with carbon

245　tape and transferred to the SEM (QuantaFEG ESEM, FEI, Eindhoven, Netherlands). The samples were

246   imaged uncoated; either at high vacuum with the Everhart-Thornley detector at 5 kV or under environmental

247   SEM (ESEM) conditions at 4.5 torr at 26°C with a Gaseous secondary electron detector at 10 kV. The latter

248   method was used for charge contrast imaging which gives rise to a material contrast based on the intrinsic

249   electrical properties of the sample components using secondary electrons. Pi-bonds in the unsaturated fatty

250   acid chains gives rise to a dynamic and tuneable contrast under ESEM conditions [19] which can be used to

251   analyse which part of the surface is actually FO [20].

252   *CLSM.* The granola bars were also subjected to imaging by confocal laser scanning microscopy (CLSM) as

253   described by Horn et al. [13]. Nile red and Fluorescein isothiocyanate (FITC) were used as the fluorescence

254   stain agents for fats and proteins, respectively. Nile red and FITC were dissolved in acetone, and a suitable

255   amount of the dye solutions were placed on a glass slide, allowing the solvents to evaporate before adding

256   the granola bar sample. Prior to that, the bars were cut to get a flat surface in order to obtain optimal contact

257   between sample and dye. CLSM was performed on a Leica TCS SP II (Leica Microsystems GmbH,

258   Heidelberg, Germany) inverted vertically, at room temperature with a 40x oil immersion objective.

259

260   *2.14.   Data treatment*

261   The results obtained were analysed by two-way ANOVA (GraphPad Prism Version 4.0, GraphPad Software,

262   Inc.). The Bonferroni multiple comparison post-test was used to test difference between samples or storage

263   time. The significance of the results is expressed as having a *p*-value < 0.05.

264   Furthermore, principal component analyses (PCA) were carried out using R studio on data retrieved from

265   samples of week 0, week 5 (except tocopherols which included data from week 4) and week 10 (except

266   volatiles which included data from week 9). Tocopherol analysis was performed every other week, thus

267   tocopherol data from week 4 were used for PCA. A drop in some volatiles was observed for AE2 and WE2,

268   thus volatiles data of week 9 were used for PCA. Standardisation and average values were used for the

269   PCAs.

270

271   **3.   Results**

272   *3.1.   Droplet size distribution, fatty acid compositions and tocopherol content*

273   The Droplet size distributions of the 70% FO-in-water emulsions were determined. The average $D[3,2]$ value

274   ranged from 0.8 to 2.4 µm. Control, WE2, AE1 and 2, EE1 and 2 showed mainly a distribution of droplets

275   from 0.8 to 1.2. However, WE1 showed a distribution of larger oil droplets of 2.4 µm.

276   The changes in the fatty acid compositions in fish-oil-enriched granola bars during 10 weeks of storage, was

277   calculated by determining the relative decreases in area% (*RDA%*) of some n-3 LC PUFA (EPA, DHA, and

278   18:4 n-3) and the total PUFAs from week 0 to 10 (Table 2). The *RDA%* of EPA, DHA and 18:4 (n-3) was

279   found to be significant ($p < 0.05$) between samples for Control and AE1. The *RDA%* of EPA and 18:4 n-3

280   was only found to be significant ($p < 0.05$) in the Control, with a decrease of 13.8% and 15.6% from week 0

281   to week 10, respectively. In contrast, *RDA%* was not significant ($p > 0.05$) for DHA or total PUFAs in any of

282   the granola bars.

283

285   Con – – –   WE1 --●--   WE2 --■--   EE1 —●—   EE2 —■—   AE1 ·······●·······   AE2 ·······■·······
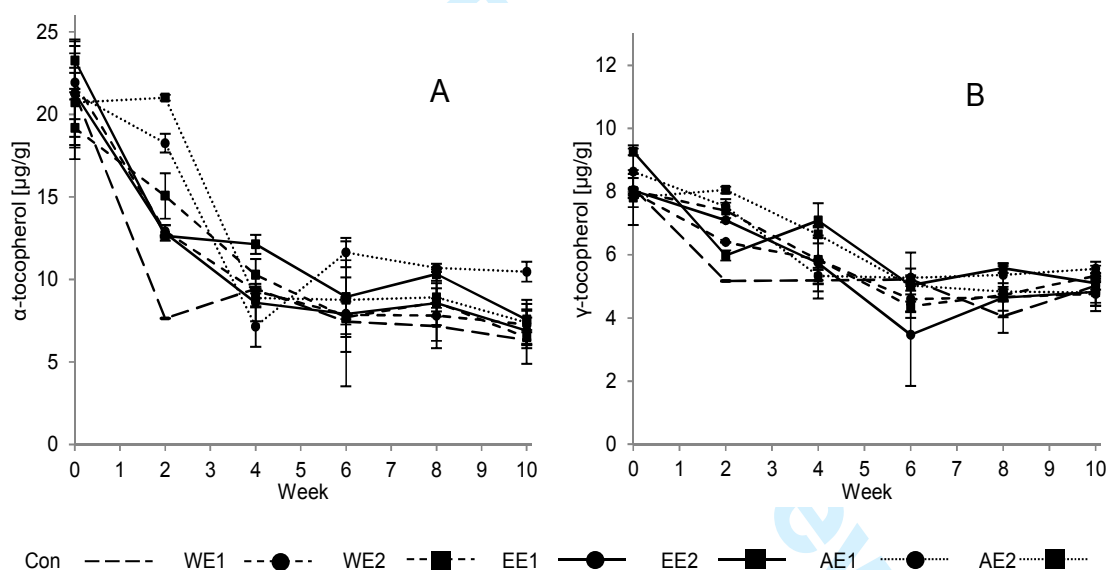
286   Fig. 1. Tocopherol content (µg/g granola bar). Development in concentrations (µg/g bar) of two tocopherol
287   homologues: (α- (A) and γ-tocopherol (B)) in FO-enriched granola bars added *F. vesiculosus* water (WE),
288   ethanol (EE) or acetone extract (AE) (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without
289   extract) stored dark for 10 weeks at room temperature (n=2).
290

291   In Fig. 1 (A-B) the content of α- and γ-tocopherols in the granola bars during storage are shown. No

292   significant consumption of δ-tocopherol during storage was found (data not shown). Consumption of α-, and

293   γ-tocopherols was observed in all samples. The consumption of α-tocopherol (70%) were larger compared to

294   γ-tocopherols (50%) at the end of storage. Comparison of the different samples showed that the fastest

295   consumption of α-tocopherol was found in Control. The α-tocopherol reduction in Control reached 65% after

296   only two weeks, whereas, at the same storage point no significant consumption of α-tocopherol was

297   observed in granola bars with high or low concentration of acetone extract added. This finding suggested

298   that the Control oxidised faster than the samples with seaweed extracts. After week 4, the consumption of α-

299   and γ-tocopherols was significant in all samples and had reached its maximum as the tocopherol content

300   reached a steady level in all samples.

301

302   *3.2.   Iron content and dry matter*

303   In Table 2, the dry matter and content of iron (Fe) and copper (Cu) of granola bars are shown. The dry

304   matter was similar in all samples (approximately 84%). The Fe content of the granola bars ranged from

305   17.2 ± 1.9 to 22.7 ± 1.7 µg Fe/g granola bar and was higher than the Cu content, which ranged from 1.7 ± 0.2 to

306   3.9 ± 1.3 µg Cu/g granola bar. There were no significant differences in either Fe or Cu content between the

307   samples.

308

309   *3.3.   Lipid hydroperoxides*

310   In Fig. 2A development of PV in the 5% FO-enriched granola bars during storage is shown. PVs ranged from

311   1.4 to 6-8 meq/kg in week 0 and 27.6 to 35.4 meq/kg in week 10. Development of PV in the granola bars

312   was somewhat different when seaweed extracts were added into the formulation compared to the control. A

313   significant increase in PV was not observed in AE2 and EE2 before week 4, whereas the other samples only

314   had a lag phase of one week. Until week 4, PV was highest in the Control. After week 4, PV was generally

315   higher in WE1 than in all other samples. Towards the end of the storage, WE1 and WE2 had the highest PV,

316   but were not significantly different from the Control. Hence, after 10 weeks of storage significant differences

317   in PV were only observed between AE1 and WE1/WE2 ($p < 0.05$) and PV of AE1 was significantly lower

318   than PV of WE1/WE2 ($p < 0.05$). However, in general EE1, EE2 and AE1 showed antioxidant activity as

319   granola bars with these extracts added had lower PVs during storage.

320

Fig. 2. Peroxide values (meq/kg oil) (A) and development of volatile secondary oxidation products (ng/g granola bar) (B-D) in FO-enriched granola bars added *F. vesiculosus* (WE), ethanol (EE) or acetone extract (AE) (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without extract) stored dark for 10 weeks at room temperature. The development of volatile secondary oxidation products exemplified by 1-penten-3-ol (B), 1-penten-3-one (C) and *t,t*-2,4-heptadienal (D). Error bars indicate SD of the measurements (n = 2 for PV and n = 3 for volatiles compounds).

3.4. Volatile secondary oxidation products

For determination of secondary oxidation products, 16 volatiles out of 100 observed volatile compounds, were identified by MS and quantified: 1-penten-3-ol, 1-pentanol, 1-octen-3-ol, pentanal, *t*-2-pentenal, *t*-2-hexenal, hexanal, *t,t*-2,4-heptadienal, 4-heptenal, octanal, nonanal, decanal, 1-penten-3-one, 2-ethylfuran, 2-pentylfuran and benzaldehyde. The identity of these volatiles was confirmed by comparison of retention times with those of external standards. Mainly, all volatile secondary oxidation products in the present study

337   showed similar development patterns in volatile concentrations during the storage period, with a lag phase

338   followed by an increase in concentration particularly in the later part of the storage period. The only

339   exceptions to this pattern were nonanal and decanal, for which the concentrations were initially high and at

340   the same level for all samples, which could the result of heat-induced oxidation in the baking phase (data not

341   shown).

342   In Fig. 2 (B, C and D), three representatives of oxidation products from EPA and DHA are shown, namely 1-

343   penten-3-one, 1-penten-3-ol and t,t-2,4-heptadienal, which have all been recognised as decomposition

344   products of EPA and DHA [21]. In addition, 1-penten-3-one has been suggested as one of the markers for

345   fishy and metallic off-flavours in fish-oil-enriched foods [22]. The length of the lag phase of the three

346   representatives differed between samples and was depending on addition and concentration of the extracts.

347   The Control (Con) had a short lag phase of 1 to 3 weeks, followed by a steep increase in concentration of

348   secondary oxidation products in the subsequent storage weeks. Samples prepared with water extract (WE1

349   and WE2) had a lag phase up to 4 weeks and hereafter had the same development as the Control. Granola

350   bars containing the high amount of ethanol extract (EE2) had the longest lag phase for volatiles up to 6 to 7

351   weeks followed by granola bars with acetone extract (AE1 and AE2). The lag phases of 1-penten-3-one were

352   generally shorter than for 1-penten-3-ol. A shorter lag phase of 1-penten-3-one than 1-penten-3-ol has been

353   observed before in determination of lipid oxidation in FO-enriched foods [23, 24, 10]. This has been found to

354   the related to the reduction of 1-penten-3-one to 1-penten-3-ol. Thus, the differences in lag phases of these

355   two compounds are the time before 1-penten-3-one has been formed and then to some extent reduced to 1-

356   penten-3-ol.

357

358   *3.5. Principal component analysis*

359   PCA was carried out to visualise and determine correlations between types of extract used in the granola

360   bars (samples) and the oxidation parameters (variables). PCAs were carried out at three different storage

361   time points (Fig. 3A, B and C).

362

Fig. 3. Principal component analysis (PCA). PCAs of week 0 (A), 5 (4 for tocopherols) (B) and 10 (9 for volatiles) (C). A, B and C are Bi-plots with FO-enriched granola bars added *F. vesiculosus* water (WE), ethanol (EE) or acetone extract (AE) in two different concentrations (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without extract) and variables: PV (peroxide value), Vol1 (1-penten-3-one), Vol2 (1-penten-3-ol), Vol3 (nonanal), Vol4 (1-pentenol), Vol5 (4-heptenal), Vol6 (2-hexenal), Vol7 (1-octen-3-ol), Vol8 (2,4-heptadienal), a-toc (alpha-tocopherol), g-toc (gamma-tocopherol), d-toc (delta-tocopherol), FS (fishy smell), RS (rancid smell), FT (fishy taste) and RT (rancid taste). For week 10 Relative Decrease in Area % (RDA%) of EPA (RDA%EPA), DHA (RDA%DHA) and PUFAs (RDA%PUFA) are included as well.

15

363   In Fig. 3A, PCA on data from week is shown. The first two principal components in this PCA explained 35

364   and 25% of the total variance in the data set, respectively. This analysis showed that rancid smell and

365   content of all tocopherols were mostly located in the right side together with the Control (Con) and EE2,

366   whereas volatiles, PV and the other sensory parameters were more located to the left together with the other

367   samples. The raw data of PV (Fig. 2A) showed that the initial PV at week 0 differed between samples but

368   this could not be distinguished in the PCA model.

369   In Fig. 3B, the PCA on data from samples of week 5 is depicted. PC1 describes 54% of the model and PC2

370   17%. At week 5, fishy and rancid taste and smell, PV and almost all volatile secondary oxidation products

371   were located to the right side together with the Control and WE1, indicating of a higher degree of oxidation in

372   these samples. This was confirmed by the raw data (Fig. 2) as these samples had higher PV and content of

373   volatile secondary oxidation products at this time point. Furthermore, EE2 and AE1 were located to the left,

374   far away from the oxidation parameters. EE2, AE2 and WE2 were located in the 3rd quarter together with the

375   tocopherols, indicating that the tocopherol contents were higher in these samples at this time point. AE1 was

376   located at the 2nd quarter far away from all the variables. The location of EE2 and AE1 indicated that these

377   two samples were the two least oxidized samples. This was also found in the raw data where PV and

378   content of volatile secondary oxidation products were lower in these two samples at this time point.

379   In Fig. 3C, the PCA on data from samples from the last week of storage is shown including the decrease in

380   EPA, DHA and total PUFAs (RDA%). The first two principal components in this PCA explained 56 and 20%

381   of the total variance in the data set, respectively. At the end of the storage the RDA% of EPA, DHA and

382   PUFAs, the volatile secondary oxidation products, PV and the sensory parameters were mostly located to

383   the right together with the Control and WE1. 1-penten-3-one (vol 1) and 1-octen-3-ol (vol 7) was located near

384   the Control, indicating higher oxidation of FO in the Control compared to other samples. This was also

385   confirmed by the raw data in Fig. 2 (B and C). AE1 was located at the 2nd quarter together with α- and γ-

386   tocopherol, indicating that this sample at the end of storage had the highest amount of these two

387   tocopherols. AE1, EE1 and EE2 were located far away from the oxidation parameters.

388   The secondary volatile compounds all moved towards the right side from week 0 to 9 ending up near the

389   Control and WE1. These results indicated that all volatiles were produced in the highest amount when no

390   seaweed extracts were added or water extract was added in the low concentration. The far lowest observed

391   content of volatiles in this study was found for AE1, EE1 and EE2, as indicated by the absence of volatiles in

392   the same quadrant as these samples in Fig. 3B and 3C.

393

394   *3.6.   Microstructure and droplet size distribution*

395   It is of great importance to investigate the lipid distribution in the granola bars in order to understand how the

396   70% FO-emulsion was incorporated in the bars. Therefore, three types of microscopy techniques were used

397   in the present study. Starch grains were found by all microscopic methods (Fig. 4) and are marked in Fig. 4.

398   (B1, B2 and A-D3). In the confocal images the starch grains were clearly distinguished in the water phase as

399   green grains with cracks in the middle. In the SEM and ESEM images the starch grains were found as

400   pointed spheres on the fracture surface of the bars and are marked in Fig. 4. (A-D3). Some of the starch

401   grains were polygons, most likely originating from the rice components. The starch grains were widely

402   distributed in the matrix both as large and smaller grains. According to the confocal images, the oil was not

403   highly associated with air (marked in Fig. 4.D1). All images showed that oil was not only found as spherical

404   oil droplets but also as large oil pools in the bars, which indicated ruptured droplets. The oil pools were most

405   pronounced in the Control where no extract was added. Examples of oil pols are marked in Fig. 4. (A1-2).

406   Among samples with extracts, the oil pools were seen more often in granola bars prepared with water

407   extract, and more evenly distributed spherical oil droplets were observed in the bars prepared with ethanol or

408   acetone extract. The final water content of the bars was low. Nevertheless, the confocal images (Fig. 4.A-

409   D1) show that the bars contained few large spherical water droplets that could be a site for lipid oxidation.

410   The confocal images also showed that protein bands were formed (light green areas). These bands

411   surrounded some of the oil droplets forming a physical barrier on the surface of the droplets, which could

412   influence the lipid oxidation. This is typically seen in protein emulsified o/w emulsions.

413

414

Fig. 4. Microstructure of the granola bars at week 0 at 20°C. A: The Control without extract, B: Granola bar added water extract (WE), C: Granola bar added ethanol extract (EE), D: Granola bar added acetone extract (AE), all with 1% extract in the FO emulsion. CLSM (1) red is the fat phase and green is the water phase, scale bars are 30 μm. ESEM (2) and SEM (3), scale bars are 20 μm and 50 μm, respectively.

420

4.   Discussion

4.1.   Influence of ingredients on lipid oxidation in granola bars

The water content was similar in all the granola bars and it was therefore assumed that the conditions and a_w

inside the granola bars was the same for all samples. Nielsen and Jacobsen [12] found the water activity (a_w)

in energy bars similar to those used in the present study to be around 0.5. The water activity of a system

influences lipid oxidation rates. In low-moisture foods the limited mobility of components like antioxidants

results in high lipid oxidation rates compared to the rate at intermediate water activities as that which can be

expected for the granola bars in the present study [25, 26].

Nielsen and Jacobsen [12] found a similar iron content (20.9 ± 0.8 µg/g) as in the present study. Iron in the

granola bars is a potential pro-oxidant in lipid oxidation since it can decompose hydrogen peroxides and lipid

hydroperoxides into free radicals [2]. This iron stems from the ingredients, such as wheat flour, raisins and

water, the process equipment, and the seaweed extracts used in this study [27, 10, 11]. Thus, iron could

contribute to the oxidation of the granola bars.

4.2.   Antioxidant efficacy of the seaweed extracts in granola bars

From the results, it was observed that the addition of water (only high concentration), ethanol or acetone *F.*

vesiculosus extract to 5% FO enriched granola bars improved the oxidative stability of the bars during

storage at room temperature. The decrease in some n-3 PUFAs was reduced by the addition of these

extracts and also the development in volatile secondary oxidation products related to oxidation of FO was

reduced to a great extent. Moreover, a decrease in volatiles in WE1, WE2 and AE2 after week 9 could

indicate further reaction of volatiles to tertiary oxidation products in these three granola bars. It seemed like

the volatiles were protected against degradation to tertiary oxidation products by ethanol extract since no

decrease in volatiles in samples added this extract was observed after week 9. The order at week 9 towards

limiting formation of volatile secondary oxidation products in the bars was as follows; AE1 > EE2 > AE2 =

EE1 = WE2 > WE1 > Control. The order was confirmed by the PCA conducted on volatiles data from week

9.

 High antioxidant efficacy of ethanol and acetone extracts from *F. vesiculosus* was also found by Honold et

al. [11] when tested in mayonnaise fortified with 15% FO. It was found that both extracts could lower the

449 formation of peroxides and increase the lag phase of the formation of some volatile compounds compared to

450 the Control. In the study of Hermund et al. [10], where similar *F. vesiculosus* water extract as in the present

451 study were tested in 5% FO enriched milk and 15% FO enriched mayonnaise, it was found that the low

452 concentration of water extract in mayonnaise showed no antioxidant effect towards the formation of

453 peroxides whereas the high concentration showed low antioxidant activity. Likewise, in the present study,

454 WE1 had high levels of most oxidation products and was located near these in the PCA. Furthermore, the

455 70% FO-in-water emulsion added 0.5 g water extract/100 g emulsion consisted of larger droplets compared

456 to the other emulsions. This made WE1 different from the other samples from the beginning and possibly

457 more susceptible to oxidation due to a higher surface area or the oil from where the lipid oxidation can occur.

458 Water extract added in the high concentration showed only weak antioxidant activity and was not as efficient

459 as the ethanol and acetone extracts. This indicates that *F. vesiculosus* water extract needs to be applied in

460 higher concentration than the ethanol extract and acetone in these foods in order to be an efficient

461 antioxidant. Moreover, the microscopy images showed that more oil was found as oil pools in granola bars

462 added no or water extract. It was expected that unprotected oil would be more pronounced to lipid oxidation

463 since emulsification creates a protective structure around the oil droplets where also antioxidants could be

464 located to hinder lipid oxidation.

465 Hermund et al. [10] and Honold et al. [11] found that water, ethanol and acetone extract of *F. vesiculosus*

466 (same extracts used in the present study) had a phenolic content of 18.4±0.1, 20.4±2.4 and 23.2±1.1 g

467 GAE/100g dry extract, respectively. The high radical scavenging activity of *F. vesiculosus* extracts has been

468 related to their high phlorotannin content, the major phenolic compound in the brown algae and thereby also

469 the total phenolic content [6, 7, 8, 10, 11]. Furthermore, polyphenols such as phlorotannins from *Fucus* sp.

470 are also good metal chelators [28]. Other ingredients such as wheat flour and oats also contribute to the

471 phenolic content in the bars and the total antioxidant activity [29].

472 EDTA has been found to be an efficient antioxidant in foods with a high content of iron, such as mayonnaise,

473 due to its metal chelating ability [3]. However, oxidation studies on energy bars and other low-moisture foods

474 such as crackers, found that metal chelators either had no antioxidant effect or exhibited prooxidant activity

475 [12, 30]. Nielsen et al. [12] found that EDTA increased oxidation in energy bars added emulsified fish oil

476 compared to when no EDTA was present. Furthermore, Barden et al. [30] studied the influence of iron on

477 lipid oxidation in low-moisture crackers added soybean oil. Based on their study they suggested that free

478   radical scavengers could be the best solution to stabilize lipids in this type of foods since they found no effect

479   of EDTA.

480   Normally, the efficacy of antioxidants is highly dependent on their location. Hermund et al. [10] and Honold et

481   al. [11] investigated the partitioning of the extracts and found low oil-water partitioning coefficient of phenolic

482   compounds ($P_{o/w}$ values: -1.3±0.0) of all extracts indicating that the phenolic compounds were mainly

483   hydrophilic and mainly located in the water phase and not in the oil phase. However, Hermund et al. [10] and

484   Honold et al. [11] also found high amounts of phenolic compounds at the interface indicating that some of the

485   phenolic compounds are amphiphilic. Thus, when the emulsion was added to the granola bar dough, the

486   phenolic compounds were presumably located both at the interface and in the water phase. At these phases

487   the antioxidants were in close proximity to lipid oxidation initiators such as iron and to some extent also the

488   radicals from the initial lipid oxidation in the oil phase. When the dough was baked, the water content and

489   mobility inside the bars decreased and the antioxidants were presumably more or less locked in these

490   positions. Moreover, oil was to a larger extent found as spherical oil droplets when ethanol and acetone

491   extract was added, indicating emulsifying properties of these extracts.

492   In the present study, consumption of α- and γ-tocopherol was observed in all bars. However, the

493   consumption rate differed between samples. The fastest initial consumption was observed in bars without

494   added any extract whereas bars added acetone extract had the slowest initial consumption. Horn et al. [13]

495   found that γ-tocopherols work efficiently as chain breaking antioxidants in energy bars added emulsified fish

496   oil, as they saw consumption of γ-tocopherol together with increased oxidative stability. Due to the lipophilic

497   nature of tocopherols, these will, after emulsification, be located inside the oil droplets or at the oil-water

498   interface and thereby close to the site of lipid oxidation. Since, phenolic compounds are able to regenerate

499   tocopherols, it can be assumed that addition of extracts, rich in amphiphilic phenolic compounds, uses their

500   radical scavenging activity for regenerating tocopherols at the oil-water interface [31]. Presumably, the

501   regeneration of tocopherols gives the high antioxidant activity in the bars added extracts, especially ethanol

502   and acetone extracts.

503

504   **5.   Conclusion**

505   Results showed that the oxidative stability of FO-enriched granola bars was improved by addition of 1 g

506   extract/100 g emulsion. Acetone extract in the low concentration and ethanol extracts in both concentrations

507 were the most efficient antioxidants in this type of food. No antioxidant activity was found for water extract

508 when applied in low concentration. Thus, concentration of the extracts seemed to have great importance to

509 the efficacy of the extracts in the bars. The antioxidant efficacy of acetone and ethanol extracts was among

510 other related high total phenolic content, high radical scavenging activity of the extracts, together with high

511 interfacial affinity of phenolic compounds. Furthermore, the microstructure of granola bars prepared with

512 ethanol and acetone extracts showed fewer oil pools and more ordered spherical oil droplets, indicating

513 emulsifying properties of the extracts and regeneration of tocopherols.

514 Since this is one of the first studies concerning lipid oxidation in low moisture foods added fish oil emulsion

515 and relating this to microstructure, further studies of emulsion optimisation and improved incorporation of the

516 fish oil in these types of products are needed.

517

6. Acknowledgement

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602   Table 1. Granola bar formulation (g/100 g)

Ingredient	Quantity
Rolled oats	15.9
Syrup	14.0
Honey	14.0
Wheat flour	11.2
Nut/fig/cocoa spread*	8.4
Rice crispies	7.0
Raisin	7.0
Apricot	7.0
Soft brown sugar	4.2
Maltodextrin	4.2
70% FO/w emulsion	7.1

603   * Hazelnuts (8.6%) were toasted and blended (Kenwood kitchen

604   blender) together with nut/fig mix (57.1%), cocoa powder (5.7%)

605   and water (28.6%).

606

607   Table 2. Dry matter (% w/w), metal content of iron (Fe) and cupper (Cu) (µg/g bar) and Relative Decrease in

608   Area % of some fatty acids (EPA, DHA, 18:4 (n-3) and PUFAs) from week 0 to 10 of FO-enriched granola

609   bars added two concentrations of extract, 0.5 (1) or 1% (2) in the FO emulsion (Control=no extract added,

610   WE=added water extract, EE=added ethanol extract, AE=added acetone extract).

Sample	Dry matter (%)	Metal content (µg/g bar)		Relative decrease in area % from week 0 to week 10			
		Fe	Cu	EPA	DHA	18:4 (n-3)	PUFAs*
Control	82.7±1.5 [a]	17.5±2.0 [a]	2.8±0.4 [a]	13.8 [a z]	11.5 [a b]	15.6 [a z]	15.1 [a]
WE1	84.0±1.3 [a]	17.8±0.0 [a]	1.8±0.1 [a]	5.2 [a b]	-5.8 [a b]	10.6 [a b]	18.5 [a]
WE2	85.7±0.9 [a]	17.2±1.9 [a]	3.6±1.8 [a]	9.1 [a b]	9.6 [a b]	7.1 [a b]	6.6 [a]
EE1	83.4±1.7 [a]	18.6±4.0 [a]	1.7±0.2 [a]	10.7 [a]	14.9 [a]	7.0 [a b]	1.1 [a]
EE2	84.3±1.2 [a]	17.6±0.2 [a]	2.0±0.3 [a]	8.4 [a b]	2.9 [a b]	8.2 [a b]	22.5 [a]
AE1	83.5±0.3 [a]	22.7±1.7 [a]	2.4±0.7 [a]	4.0 [b]	-3.2 [b]	6.9 [b]	12.8 [a]
AE2	83.9±0.7 [a]	21.9±1.4 [a]	3.9±1.3 [a]	9.7 [a b]	11.2 [a b]	7.6 [a b]	5.2 [a]

611   a, b, c: significance between samples within the same column

612   z: significant decrease (from week 0 to 10) in EPA, DHA, 18:4 (n-3) or PUFAs in the sample

613   *PUFAs: total of C18:2 (n-6), C18:3 (n-3), C18:4 (n-3), C20:4 (n-3), C20:5 (n-3, EPA) and C22:6 (n-3, DHA)

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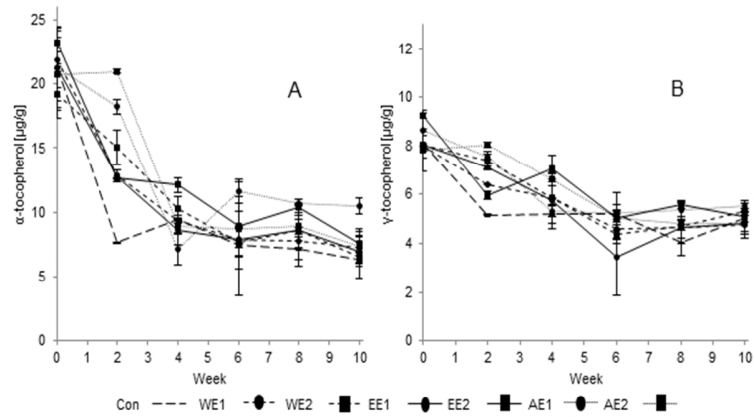
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Fig. 1. Tocopherol content (µg/g granola bar). Development in concentrations (µg/g bar) of two tocopherol homologues: (α- (A) and γ-tocopherol (B)) in FO-enriched granola bars added F. vesiculosus water (WE), ethanol (EE) or acetone extract (AE) (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without extract) stored dark for 10 weeks at room temperature (n=2).
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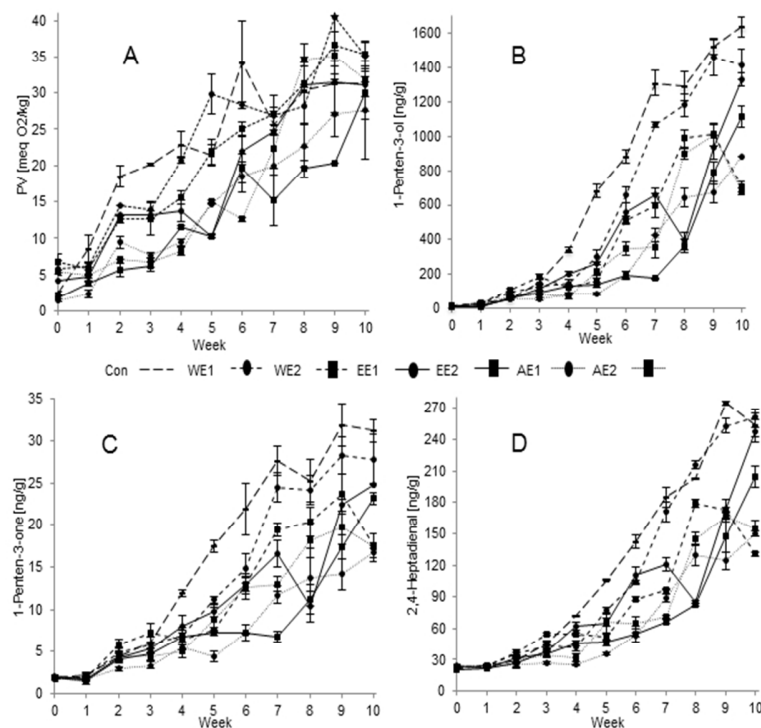
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Fig. 2. Peroxide values (meq/kg oil) (A) and development of volatile secondary oxidation products (ng/g granola bar) (B-D) in FO-enriched granola bars added F. vesiculosus (WE), ethanol (EE) or acetone extract (AE) (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without extract) stored dark for 10 weeks at room temperature. The development of volatile secondary oxidation products exemplified by 1-penten-3-ol (B), 1-penten-3-one (C) and t,t-2,4-heptadienal (D). Error bars indicate SD of the measurements (n = 2 for PV and n = 3 for volatiles compounds).
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Fig. 3. Principal component analysis (PCA). PCAs of week 0 (A), 5 (4 for tocopherols) (B) and 10 (9 for volatiles) (C). A, B and C are Bi-plots with FO-enriched granola bars added F. vesiculosus water (WE), ethanol (EE) or acetone extract (AE) in two different concentrations (0.5 (1) or 1% (2) in the FO emulsion) and a Control (Con) (without extract) and variables: PV (peroxide value), Vol1 (1-penten-3-one), Vol2 (1-penten-3-ol), Vol3 (nonanal), Vol4 (1-pentenol), Vol5 (4-heptenal), Vol6 (2-hexenal), Vol7 (1-octen-3-ol), Vol8 (2,4-heptadienal), a-toc (alpha-tocopherol), g-toc (gamma-tocopherol), d-toc (delta-tocopherol), FS (fishy smell), RS (rancid smell), FT (fishy taste) and RT (rancid taste). For week 10 Relative Decrease in Area % (RDA%) of EPA (RDA%EPA), DHA (RDA%DHA) and PUFAs (RDA%PUFA) are included as well. 254x190mm (96 x 96 DPI)
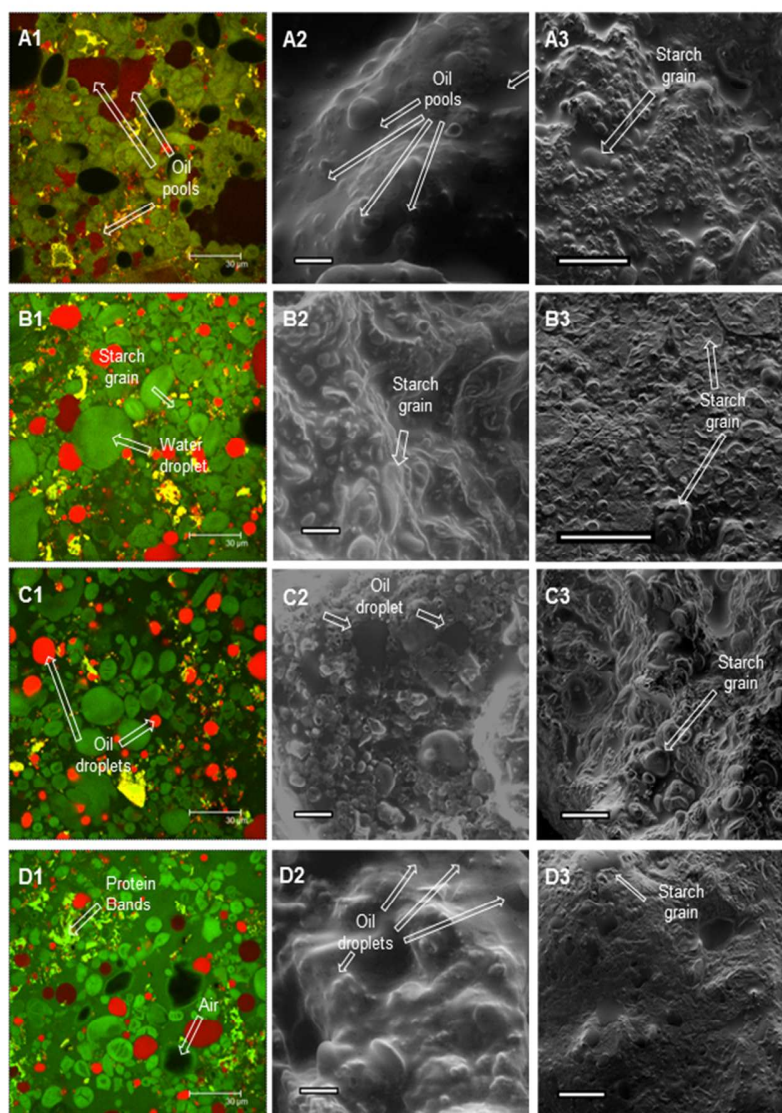
Fig. 4. Microstructure of the granola bars at week 0 at 20°C. A: The Control without extract, B: Granola bar added water extract (WE), C: Granola bar added ethanol extract (EE), D: Granola bar added acetone extract (AE), all with 1% extract in the FO emulsion. CLSM (1) red is the fat phase and green is the water phase, scale bars are 30 μm. ESEM (2) and SEM (3), scale bars are 20 μm and 50 μm, respectively.
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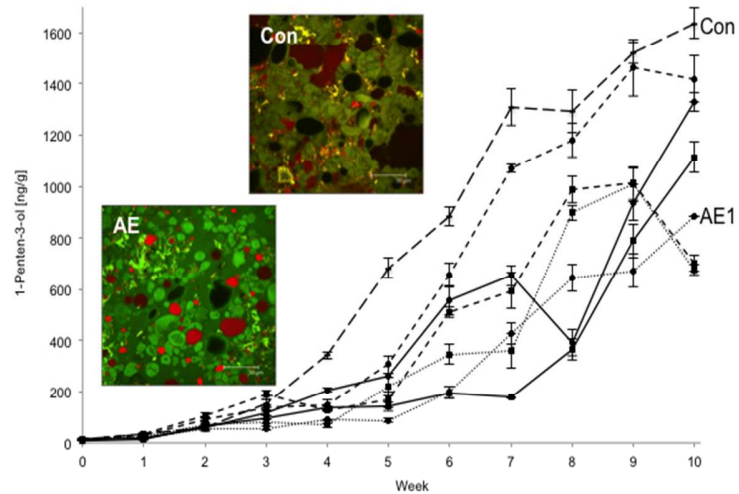
Graphical abstract. Granola bars enriched with 10% Na-Cas fish oil-in-water emulsion where *F. vesiculosus* extracts where added as antioxidant to control lipid oxidation. Development of secondary oxidation product, 1-penten-3-ol (ng/g granola bar) in the granola bars and confocal light scattering microscopic images of granola bar added 0.5 g acetone extract/100g emulsion (AE1) and the Control (Con) (without extract). The efficacy of acetone extracts to limit development of 1-penten-3-ol in granola bars was found to be related to extract composition as well as the microstructure, as fish oil emulsions in the bars were more stable when this extract was added. Error bars indicate SD of the measurements (n = 2 for PV and n = 3 for volatiles compounds).

PAPER IV

Oxidative Stability of Granola Bars Enriched with Multilayered Fish Oil Emulsion in the presence of Novel Brown Seaweed Based Antioxidants. Journal of Agricultural and Food Chemistry

Hermund, DB, Karadağ, A, Andersen, U, Jónsdóttir, R, Kristinsson, HG, Alasalvar, C, Jacobsen, C

1 **Oxidative Stability of Granola Bars Enriched with Multilayered Fish Oil Emulsion in the**

2 **presence of Novel Brown Seaweed Based Antioxidants**

3

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20 Key words: *Fucus vesiculosus*, Omega-3, multi-layered emulsion, CLSM

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22

23 **Abstract**

24 The oxidative stability of granola bars added fish oil emulsions, either as primary and secondary

25 emulsion systems stabilized by sodium caseinate and sodium caseinate-chitosan. *Fucus vesiculosus*

26 extracts were added to the granola bars to their antioxidant activity in these systems. The bars were

27 stored at 20°C and evaluated over a period of 10 weeks by measuring the development of primary

28 and secondary oxidation products. The microstructure of the granola bars were determined by

29 confocal light scattering microscopy (CLSM).

30 Additional protection against lipid oxidation was obtained when multilayer fish oil emulsions were

31 added to the granola bars especially in combination with acetonic (AE) and ethanolic (EE) extract.

32 The results from this study indicated the importance of the presence of high levels of phlorotannins

33 in the extracts, thus AE and EE can be recommended for application in these types of products,

34 even at the low concentrations tested (0.4 g dw/kg product).

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1. Introduction

Lipid oxidation in foods has been counted as a major problem due to its detrimental effects on the quality of food including formation of a wide range of unpleasant odours and rancid taste. The oxidative stability of foods highly depends on their composition such as the degree of unsaturation of the lipids, and it can be affected by many factors such as heat, accessible oxygen and the presence of pro-oxidants and antioxidants, which affect the initiation, and the rate of oxidation. During the last couple of decades, there has been an increasing demand for functional foods enriched with n-3 long chain polyunsaturatated acid (LC PUFA) of marine origin, especially docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) due to their health benefits (Horrocks and Yeo, 1999; Covert, 2009). Although fortification of processed foods with n-3 LC PUFAs has been considered one of the most effective ways to increase n-3 LC PUFAs intake and improve health, there are considerable challenges to incorporating n-3 FAs into many types of functional food products due to their susceptibility to lipid oxidation that would lead to formation of degradation products, off-flavours and other sensory alterations, reduce the acceptance of the functional product by consumers (Frankel 2005; Walker et al., 2015).

Although, synthetic antioxidants, e.g. butylated hydroxytolulene (BHT) and ethylenediaminetetraacetic acid (EDTA), are extensively used to hinder lipid oxidation in foods, some restrictions have been made on their application in food due to health risks and possible toxicity issues (Branen, 1975; Linderschmidt et al., 1986). Moreover, synthetic antioxidants are in many cases not able to prevent oxidative flavour deterioration in fish oil enriched foods (Jacobsen et al., 2008). Hence, not only due to insufficiency and safety issues but also to the consumers' increasing demands for natural sustainable ingredients, there are emerging interests in replacing synthetic antioxidants with natural plant-based alternatives. In addition to the use of antioxidants,

63   the oxidative stability of emulsified oil can also be increased by controlling emulsifier type,

64   location, and concentration (Grigoriev & Miller, 2009). Creating thick interfacial layers around

65   emulsion droplet membranes that hinder interactions between water soluble prooxidants and lipids

66   inside the emulsion droplet may also inhibit lipid oxidation. For example, when oil-in-water

67   emulsion droplets are surrounded by cationic emulsifiers, prooxidant metals are repelled and lipid

68   oxidation rates decrease (Klinkesorn et al., 2005; Shaw et al., 2007; Jiménez-Martín et al., 2015).

69

70   Although in the study of Klinkesorn et al. (2005), the lipid oxidation during storage was slower in

71   the secondary emulsions stabilized by lecithin-chitosan as compared to primary emulsions produced

72   by lecithin alone, oxidation markers were still observed to increase over time. Therefore, the

73   presence of antioxidants is needed to further decrease the lipid oxidation. Antioxidants originating

74   from marine biomass have high potential for becoming the next generation of natural antioxidants.

75   Brown algae, *Fucus vesiculosus*, or more popularly called bladderwrack, is rich in the bioactive

76   polyphenolic secondary metabolites, *phlorotannins* (Chkhikvishvili & Ramazanov, 2000; Wang et

77   al., 2009, 2010 & 2012). Studies of these compounds extracted from *F. vesiculosus* have shown that

78   they possess both radical scavenging activity and metal chelating ability *in vitro*, which makes them

79   well-suited as antioxidants in foods. Hermund et al. (2015) and Honold et al. (2015) found that

80   different extracts of Icelandic *F. vesiculosus* showed not only excellent *in vitro* antioxidant

81   properties but also a great potential as natural antioxidants in emulsified foods such as milk and

82   mayonnaise enriched with fish oil. However, the effects of antioxidants cannot be extrapolated from

83   one product to another, but must be evaluated in each case, as the efficacy of the same antioxidant is

84   highly dependent on product composition and process (Nielsen et al. 2004; Jacobsen et al. 2008).

85   The studies related to clarify how oxidative stability can be maintained in solid functional food

86   matrices fortified by highly unsaturated fish oil are very scarce. In the study of Nielsen and

87   Jacobsen (2009), the delivery system that was best suited for the introduction of fish oil to the

88   energy bars was investigated and found that the bars enriched with fish oil emulsions were more

89   stable to lipid oxidation than the bars enriched with neat fish oil. In a follow up study, selected

90   antioxidants with hydrophilic (caffeic), amphiphilic (ascorbyl palmitate) and lipophilic (tocopherol)

91   properties were added to the energy bars enriched with fish-oil-emulsion. It was found that only the

92   lipophilic antioxidant tocopherol reduced lipid oxidation when it was added above 440 µg/g fish oil,

93   and other antioxidants (caffeic acid and ascorbyl palmitate) showed prooxidative effects when

94   tested in concentrations from 75 to 300 µg/g fish oil (Horn et al. 2009).

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96   In this study, we introduced fish oil into the granola bars by using primary and secondary emulsion

97   systems stabilized by sodium caseinate and sodium caseinate-chitosan in the presence of *F.*

98   *vesiculosus* extracts to investigate the ability of extracts in the delivery sytems to inhibit lipid

99   oxidation. The oxidative stability of the bars at 20°C was evaluated over a period of 10 weeks by

100   measuring the development of peroxides and volatile compounds using dynamic headspace gas

101   chromatography mass spectrometry (DHS GC-MS) together with expert sensory panel. The

102   incorporation of the fish-oil-emulsions and the physical microstructure of the granola bars were

103   determined by confocal light scattering microscopy (CLSM).

104

105   **2.   Material and methods**

106   *2.1. Materials*

107   The ingredients included soft brown sugar and syrup (Dansukker, Nordic Sugar, Denmark), honey

108   (Jakobsen A/S, Denmark), wheat flour (Flechtorfer Mühle GmbH, Germany), rolled oats

109   (Lantmännen Cerealia A/S, DK), Kellogg's Rice Crispies (Nordisk Kelloggs ApS, Denmark),

110   raisins (K.F.C. Gida A/S, Turkey), apricots (Elmas Dis Ticaret A/S, Turkey), and fig/cocoa spread:

nut/fig mix (Castus A/S, Denmark), hazelnuts and cocoa powder without added sugar (Coop, Denmark), and water. All ingredients were purchased from the local supermarket. Sodium caseinate was provided by Arla (Arla Foods Ingredients amba, Viby J., Denmark), maltodextrin (Viking nutrition, NK Import, Denmark), and fish oil (Maritex 43-01) by Maritex (Maritex, Sortland, Norway). Food grade chitosan (DDA: 91.3%; insolubilities= 0.35%, moisture=8.7%, ash= 0.78%, viscosity at pH 4.1= 12 cps) was kindly donated from G.T.C Bio Corporation (China). The fish oil, used in the emulsions for the bars, had a peroxide value (PV) of 0.854 meq peroxides/kg oil and a tocopherol content of 418 µg α-tocopherol/g oil, 0 µg β-tocopherol/g oil, 192 µg γ-tocopherol/g oil and 62 µg δ-tocopherol/g oil. The fatty acid composition of fish oil used in the study (only the content $\geq 0.5\%$) was as follows: 14:0 (2.5 %); 16:0 (10.3 %); 16:1 (n-7) (6.5 %) ; 18:0 (3 %); 18:1 (n-9) (24.8 %); 18:1 (n-7) (3.6 %); 18:2 (n-6) (9.5 %); 18:4 (n-3) (0.9 %); 20:1 (n-11+n-9) (10 %); 20:1; 20:4 (n-3) (0.5 %); 20:5 (n-3) (EPA) (6.2 %); 22:1 (n-11) (4.6 %) and 22:6 (n-3) (DHA) (7.6 %). All solvents used were of HPLC grade and purchased from Lab-Scan (Dublin, Ireland). Internal standards were purchased from Sigma Aldrich (Steinheim, Germany).

2.2. Extract production from Icelandic Fucus vesiculosus

Extraction was performed by Matís in Iceland according to Wang et al. (2009; 2012). The *F. vesiculosus* was collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in 2011. At the collecting spot the seaweed was washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water, wiped with paper towel. They were then freeze-dried, pulverised into powder and stored at -80 °C prior to extraction.

The water, acetone and ethanol extracts were produced as follows: Five grams of the algal powder was mixed with either, 100 mL of distilled water, 70 % aqueous acetone (v/v) or 80 % ethanol

135 (v/v), and incubated in a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ)

136 for 24 h at 124 g at room temperature. The mixture was centrifuged at 2168 g for 10 min at 4 °C

137 and filtered with Whatman no. 4 filter paper. Each extraction was conducted in duplicate. The water

138 (WE), acetone (AE) and ethanol (EE) extract were stored at -80 °C until used for application.

139 The composition of the seaweed extracts was determined and reported in Hermund et al. (2015) and

140 Honold et al. (2015). The composition of potential antioxidant or prooxidant compounds in the

141 extracts is given in Table 1.

142

143 *2.3. Production of FO-in-water emulsions*

144 **Solution Preparations**: The desired concentrations of sodium caseinate (Na-cas) were prepared by

145 dispersing Na-cas powder into 0.02 M phosphate buffer (pH 5.5) under gentle stirring at room

146 temperature for ~6 h until complete solubilisation, and the dispersions were stored at 4 °C overnight

147 to allow complete hydration. Chitosan solutions were prepared by dispersing weighed amounts of

148 powdered chitosan 0.02 M into acetate buffer solution (pH 5.5) and stirred overnight to ensure

149 complete hydration.

150 **Emulsion Preparations:** Coarse emulsion was prepared by adding slowly fish oil to Na-cas solution

151 while vigorously mixing with an ultra-turrax at approximately 10000 rpm until all fish oil was

152 added. To produce the primary emulsion, the coarse emulsion was homogenized by using Niro

153 Soavi table homogenizer at 250 bar by three passes. Chitosans are positively charged at low pH

154 values (pKa 6.3-7) and can be used to cover the negatively charged surfaces of Na-Caseinate which

155 has negative charges above its pI point (~4.6). Secondary emulsions were prepared by mixing the

156 primary emulsion (50% oil, 1.2 % Na-Cas) with chitosan solutions (0-1 w/v %) at 1:1 (w:w) weight

157 ratio for 2 hours at room temperature, any flocs formed in this secondary emulsion were disrupted

158 by one passage through the homogenizer at 100 bar. All emulsions (primary and secondary) added

159 into the dough of granola bars contained 25 wt % oil, 0.6 % Na-cas. When the emulsions were

160 prepared by seaweed extracts, they dissolved in Na-cas solution having 0.2 g extract in 100 g final

161 emulsion.

162

163 *2.4.   Droplet size distribution*

164 The size of the oil droplets in emulsions was determined using a Mastersizer2000 (Malvern

165 Instruments, Worcestershire, UK). The Mastersizer2000 was set up with a refractive index of

166 material of 1.4694 (oil) and of dispersant of 1.3333 (water). The droplet size was measured by

167 dissolving emulsion in The samples were dissolved first in phosphate buffer (1:10, w:w 0.02 M pH

168 5.5) and added drop wise directly into the recirculating water until an obscuration between 12-17%

169 was reached at 3000 rpm. The droplet size was measured three times. The average of the

170 measurements was taken for further calculation. Results were expressed in $D[3,2] = \sum d^3/d^2$

171 (Rawle, 1996).

172

173 *2.5.   Zeta potential measurements*

174 The zeta potential of emulsion droplets was measured using a Zetasizer Nano (Malvern Instruments,

175 LTd. Malvern, UK). Samples were diluted 100 times with the phosphate buffer (0.02 M pH 5.5).

176 The average of five recordings on three separate samples is reported as zeta potential.

177

178 *2.6.   Production of 5% FO-enriched granola bars and sampling*

179 The production of granola bars was carried out according to Horn et al. (2009) with some

180 alterations. The ingredients were weighted according to Table 2. Firstly, the nut/fig/cocoa spread

181 was prepared. Hazelnuts were toasted and blended (Kenwood kitchen blender) together with nut/fig

182 mix, cocoa powder and water to a smooth paste. All dry ingredients; wheat flour, rolled oats,

183 maltodextrin, rice crispies, raisins and apricots (chopped finely by hand) were mixed. Separately, all

184 wet ingredients; soft brown sugar, syrup, honey, nut/fig/cocoa spread and the FO emulsion were

185 mixed. Subsequently, the wet mixture was poured onto the dry ingredients. Final mixing was

186 carried out on a Viking mixer (20PS, Seidefors Agenturer AB, Bandhagen, Sweeden) for 1 min at

187 speed 60. The dough was baked in tin foil trays (500 g volume) with 350 g in each tray (equals one

188 batch) for 15 min at 175°C. After cooling at room temperature, each batch was cut into portions of

189 35 g for both chemical and sensory analysis. The granola bars was packed in sealed plastic bags at

190 atmospheric conditions and stored at room temperatures (Average 21 °C) in the dark for up to 10

191 weeks. Dry matter (%) of bars was determined on week 0 samples and given in Table 4.

192

193 *2.7. Iron and Cupper content*

194 The samples were destructed prior to metal analysis: 0.5 g sample were mixed with 5 mL HNO_3

195 (68%), 3 mL H_2O_2 (30%) and 0.5 mL HCl (37%). Two reference samples with fish protein certified

196 reference material for trace metals (DORM-3) (National Research Council, Ottawa, ON, Canada)

197 were also prepared. Destruction was carried out in a destruction oven (Multiwave 3000, Anton Paar,

198 Graz, Austria), in which the samples were heated for 10 min at 1400 W. After destruction, the

199 samples were kept at room temperature for 20 min before being transferred into 10 mL volumetric

200 flasks and filled with water. Prior to analysis, samples were diluted to reach acidity below 2%

201 HNO_3 and 1% HCl. Analysis was performed using Inductively Coupled Plasma Mass Spectrometry

202 (ICPMS) (ICPMS model 7500ce, Agilent Technologies, Wilmington, DE, USA) equipped with a

203 Scott-type spray-chamber and a concentric nebulizer with a CETAC autosampler (ASX-500,

204 CETAC Technologies, Omaha, NE, USA). The analysis was done on the isotopes 57Fe and 63Cu

205 with the ICPMS in collision-reaction-cell (CRC) mode using helium as cell-gas for interference

206 reduction. The calibration was done using external calibration with standard solutions in the

207 concentration range from 0-200 µg/L for both elements. The calibration standards were prepared by

208 dilution of certified single-element stock solutions (SCP Science, Courtaboeuf, France) of 1000

209 mg/L. The analysis was performed in duplicates.

210

211 *2.8. Extraction of lipids*

212 Lipids were extracted with a homogeneous mixture of chloroform, methanol, and water (2:2:1.8),

213 following the method of Bligh and Dyer (1959). The method was modified to use a smaller volume

214 of solvents as described by Iverson et al. (2001), but the original ratio between chloroform,

215 methanol, and water was maintained. Five grams of sample was used for lipid extraction. The lipid

216 extracts were used for the subsequent determination of fatty acid composition and lipid content. The

217 lipid content was determined by gravimetry after evaporation of chloroform. Duplicate analyses of

218 each sample were performed.

219

220 *2.9. Fatty acid composition (fatty acid methyl esters, FAME)*

221 The fatty acid composition of the oil phases was determined after fatty acid methylation and

222 analysis by GC-FID. 100 µL toluene, 200 µL heptane with 0.01 % (v/v) BHT and 100 µL internal

223 standard (C23:0) (2 % w/v) were added to an amount of lipid extract corresponding to 30-60 mg

224 lipid. One mL of BF3 in methanol was added to the lipid extract mixture and the lipids were

225 methylated in a one-step procedure using a microwave oven (Multiwave3000 SOLV, Anton Paar,

226 Graz, Austria) with a 64MG5 rotor. The settings for the microwave were 5 min at 500 Watt

227 followed by 10 min cooling. The fatty acid methyl esters (FAMEs) were washed with 1 mL

228 saturated NaCl and 0.7 mL heptane with 0.01 % (v/v) BHT. The heptane phase was transferred to a

229 GC vial and FAMEs were analysed by GC (HP 5890A, Agilent Technologies, Palo Alto, CA, USA)

230 according to AOCS (1998). For separation DB127-7012 column (10 m x ID 0.1 mm x 0.1 µm film

231 thickness, Agilent Technologies, Palo Alto, CA, USA) was used. Injection volume was 0.2 μL in

232 split mode (1:50). The initial temperature of the GC-oven was 160°C. The temperature was set to

233 increase gradually being as follows: 160 -200 °C (10.6 °C/ min), 200 °C kept for 0.3 min, 200 -

234 220 °C (10.6 °C/ min), 220 °C kept for 1 min, 220 - 240 °C (10.6 °C/ min) and kept at 240 °C for

235 3.8 min. The determination was made in duplicates. Results were given in area %.

236

237 *2.10. Tocopherol content*

238 The lipid extracts were (after evaporation under nitrogen) dissolved in heptane and analysed by

239 high-performance liquid chromatograph (HPLC) (Agilent 1100 Series, Agilent Technology, Palo

240 Alto, CA, USA) according to AOCS (1998) to quantify the content of α-, β-, γ- and δ-tocopherol in

241 the samples. These tocopherol homologues were separated using a silica column (Waters (Dublin,

242 Ireland), 150 mm, 4.6 mm, 3 μm silica film). A stock solution containing known amounts of α-, β-,

243 γ- and δ-tocopherol was prepared and used for quantification. Samples were analysed at week 0, 2,

244 4, 6, 8 and 10. The analysis was performed in duplicates and results were reported as μg

245 tocopherol/g granola bar.

246

247 *2.11. Primary oxidation products, PV*

248 PVs of the lipid extracts were determined according to the method by Shantha and Decker (1994),

249 based on the formation of an iron-thiocyanate complex. The coloured complex was measured

250 spectrophotometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific Instruments, Columbia,

251 MD). Samples were analysed at each sampling point from week 0 to 10. The analysis was

252 performed in duplicate and the results are expressed in milli-equivalents peroxides/kg oil (meq

253 O_2/kg oil).

254

2.12. Secondary oxidation products, volatiles

The collection of the volatile compounds was carried out using approximately 4 g of crushed

granola bar sample (including addition of 30 mg internal standard, 4-methyl-1-pentanol) mixed with

25 mL distilled water. The volatile secondary oxidation products were collected at 45ºC by purging

with nitrogen for 30 min at 340 mL/min. Hereafter the tubes were flushed with nitrogen (flow of 50

mL/min) for 20 min to remove water from the Tenax GR^{TM} tube. The trapped volatiles were

desorbed from the Tenax tubes by heat (200 °C) using an automatic thermal desorber (ATD-400 ,

Perkin Elmer, Norwalk, CT), cryofocused on a cold trap (-30 °C), released again (220 °C), and led

to a GC an Agilent 5890 IIA model (Palo Alto, CA, USA) equipped with a HP 5972 mass selective

detector. Chromatographic separation of volatile compounds was performed on a DB1701 column

(30 m x ID 0.25 mm x 0.5 μm film thickness, J&W Scientific, Folsom, CA) using helium gas flow

(1.3 mL/min). Initial oven temperature was 45°C, rising by 2.0°C /min to 80°C followed by an

increase of 3.0 °C /min to 150 °C and finally increased by 12.0 °C/min to 240 °C. The individual

compounds were analysed by mass spectrometry (MS) (HP 5972 mass-selective detector; Hewlett

Packard), identified by MS-library searches and addition of the internal standard, and quantification

through calibration curve made by adding the standards directly on the Tenax tubes as described in

Nielsen et al. (2007). For the quantification, a stock solution of 16 volatiles was prepared and a

calibration curve was conducted in a range from 0-2 mg/g. The analysis was carried out in

triplicates and results are given as concentration (ng/g granola bar).

2.13. Sensory evaluation

A preliminary sensory evaluation was carried out by an expert panel (minimum three persons),

rating the fish-oil-enriched granola bars from 0-10, where 0 was "none" (attributes not detectable)

and 10 was "very much" (high intensity of the attributes). The evaluation was made based on six

279 sensory parameters (fishy odour and taste, rancid odour and taste, or sweet/brown sugar odour and

280 taste) according to Horn et al (2009). The granola bars were given a code (four ciphers) and were

281 served in a randomised order. Prior to the evaluation the granola bars where cut into three or four

282 equally sized portions and served at room temperature on a tin foil tray. The expert panel evaluated

283 the samples and decided on a common rating of each sample (no statistics applied). Between each

284 tasting the panel cleansed their mouth with water.

285

286 *2.14. Microscopy*

287 Confocal laser scanning microscopy (CLSM) performed as described by Horn et al. (2009) on week

288 0 samples with added neat fish oil, primary and secondary fish oil emulsions that all samples

289 included sea weed ethanol extracts Nile red and Fluorescein isothiocyanate (FITC) were used as

290 the fluorescence stain agents for fats and proteins, respectively. Nile red and FITC were dissolved

291 in acetone, and a suitable amount of the dye solutions were placed on a glass slide, allowing the

292 solvents to evaporate before adding the granola bar sample. Prior to that, the bars were cut to get a

293 flat surface in order to obtain optimal contact between sample and dye. CLSM was performed on a

294 Leica TCS SP II (Leica Microsystems GmbH, Heidelberg, Germany) inverted vertically, at room

295 temperature with a 40x oil immersion objective

296

297 *2.15. Statistics*

298 The results obtained were analysed by two-way ANOVA (SPSS, IBM Software, Inc.). The Tukey

299 HSD multiple comparison post-test was used to test difference between samples or storage time.

300 The significance of the results is expressed as having a *p*-value < 0.05.

301 Furthermore, principal component analyses (PCA) where carried out using R studio on data

302 retrieved from samples of week 0, week 5 (except tocopherols which included data from week 4)

303 and week 10. Tocopherol analysis were done only every other week and there was no tocopherol

304 data of week 5, thus tocopherol data from week 4 were used here. Standardisation and average

305 values were used for the PCAs.

306

307 **3.   Results and Discussion**

308 *3.1.   Formation of primary and secondary emulsions*

309 The particle size and surface charge of droplets in the primary and secondary emulsions were given

310 in Table 3. Addition of extracts increased the particle size in both primary and secondary emulsions,

311 where it was more pronounced in emulsions with acetone and ethanol extracts. Neither of extracts

312 changed the surface charge of primary nor secondary emulsion droplets compare to the blank

313 samples with no extracts.

314 As in seen in Figure 1 the surface charge of droplets was changed from negative to positive when

315 chitosan was added to primary emulsion, and the initial charge of anionic droplets increased from -

316 25 mV to approximately +21 mV with the addition of chitosan (0.5 w/v%). The surface charge

317 became close to zero after addition of 0.1 w/v% suggesting that around this chitosan concentration

318 charge neutralization occurred. Both the particle size and surface charge depended on the chitosan

319 concentration added. The size of anionic droplets was around 0.45 μm and, phase separation and

320 formation of a supernatant and a sediment layer at the bottom of test was occurred when chitosan

321 concentration was up to 0.2 w/v%. This has been explained by the fact that insufficient amount of

322 polymer was present causing a single polymer chain to attach simultaneously to 2 or more droplets,

323 and formation of such polymer "bridges" yields very large aggregates that sediment rapidly.

324 Previous studies have similarly indicated an increased likelihood for bridging flocculation to occur

325 when charges transition from net negative to net positive or vice versa (Laye et al., 2008). Above a

326 critical concentration windows, the mean diameter decreased again with increasing chitosan

327   concentration (>0.2 w/v%). The mean diameter was lowest (0.9 μm) at a chitosan concentration of

328   approximately 0.5 (w/v%), where the ζ-potential had reached a constant value where no phase

329   separation was visible in test tubes.

330

331   *3.2.   Fatty acid compositions and tocopherol content*

332   The changes in the fatty acid compositions in fish-oil-enriched granola bars during 10 weeks of

333   storage, was given by determining the relative decreases in area% (*RDA%*) of some n-3 LC PUFA

334   (EPA, DHA, and 18:4 n-3) and the total PUFAs from week 0 to 10 (Table 4). All samples except

335   EM2E showed decrease in fatty acids *RDA%* values, and the difference among other samples were

336   not significant (p> 0.05).

337   The water content was similar in all the granola bars (Table 4), and it was therefore assumed that

338   the conditions and a_w inside the granola bars was the same for all samples. Nielsen and Jacobsen

339   (2009) found the water activity (a_w) in energy bars similar to those used in the present study to be

340   around 0.5. The water activity of a system influences lipid oxidation rates. In low-moisture foods

341   the limited mobility of components like antioxidants results in high lipid oxidation rates compared

342   to the rate at intermediate water activities as that which can be expected for the granola bars in the

343   present study (Labuza et al., 1972; Nelson & Labuza, 1992).

344

345   All samples had similar iron content ranged from 18.9±2.2 to 25.1±0.1μg Fe/g granola bar and, it

346   was higher than Cu content, which ranged from 2.0±0.2 to 3.7±1.3 μg Cu/g granola bar, and there

347   were no significant differences in either Fe or Cu content between the samples. Metal ions in the

348   granola bars are potential pro-oxidants in lipid oxidation since they can decompose hydrogen

349   peroxides and lipid hydroperoxides into free radicals (Frankel 2005). They stem from the

350   ingredients, such as wheat flour, raisins and water, the process equipment, and the seaweed extracts

351 used in this study (Yeung et al., 2003; Hermund et al., 2015; Honold et al., 2015). Thus, their

352 presence could contribute to the oxidation of the granola bars.

353

354 In Figure 2 (A-B) the content of α- and γ-tocopherols in the granola bars during storage are shown.

355 No significant consumption of δ-tocopherol during storage was found (data not shown).

356 Consumption of α-, and γ-tocopherols was observed in all samples, where the largest consumption

357 was observed for α-tocopherol (~50%) compared to γ-tocopherols (~25%) at the last weeks of

358 storage. The highest consumption of α-, and γ-tocopherols was observed from week 0 to week 6 in

359 all samples. Comparison of the different samples showed that samples prepared either primary or

360 secondary emulsions showed similar reduction in terms of tocopherol values.

361

362 *3.3. Lipid hydroperoxides*

363 In Figure 3A development of PV in the fish-oil enriched granola bars during storage was shown.

364 PVs ranged from 1.3 to 2.1 meq/kg in week 0 and 26.5 to 56.5 meq/kg in week 10. PV values of

365 primary and secondary emulsions were given in Table 3. Although PV values of blank secondary

366 emulsions were higher than primary emulsion, the difference was not significant ($p > 0.05$). PV

367 values of emulsions with seaweed extracts were significantly higher than blank samples in both

368 primary and secondary emulsions. This can be explained that, addition of seaweed extracts made

369 the emulsion more viscous that possibly due to the association of the extracts with oil/water

370 interfaces. Hermund et al. (2015) and Honold et al. (2015) also found high amounts of phenolic

371 compounds at the interface indicating that some of the phenolic compounds are amphiphilic.

372 Moreover, coarse seaweed acetone, ethanol and water extracts were not only compose of

373 polyphenols, but also protein and polysaccharides that might interfere in the droplet surfaces and

374 emulsifiers used in the delivery system, and increased viscosity of the emulsion resulted in higher

375   temperature increase in homogenizer, concurrently gave higher PV values. However, initial PV

376   values of the granola bars prepared by the addition of first and secondary emulsions and baking at

377   175°C for 15 min did not show significant differences in terms of having seaweed extract or not.

378

379   Among all granola bars, the samples prepared by secondary emulsions showed lower PV values

380   compared to samples prepared by primary emulsions whether having seaweed extracts or not.

381   Between the samples prepared by primary emulsions, EM1A had lower PV values than others by

382   week 4, afterwards all samples had similar PV values. Between the samples prepared by secondary

383   emulsions, EM2E had lower PV values than others by week 5 and it was followed by EM2A.

384   When Klinkesorn et al (2005) found that fish oil in water emulsion droplets coated by lecithin and

385   chitosan produce cationic droplets that are more stable oxidation than emulsions coated by anionic

386   lecithin alone. They suggested that, the positively charged droplets in the secondary emulsion

387   inhibited iron-lipid interaction presumably decreasing the ability of iron to interact with emulsion

388   interface through electrostatic repulsion. They also suggested that, the greater oxidation rates in

389   primary anionic emulsions could be due to increased interfacial iron concentration because of

390   attractive forces. The thickness of interfacial layer is not only important to protect structure against

391   coalescing but also may protect lipids from oxidation by acting as a barrier to the penetration and

392   diffusion of molecular species that promote oxidation.

393

394   *3.4. Volatile secondary oxidation products*

395   For determination of secondary oxidation products,16 volatiles out of 100 observed volatile

396   compounds, were identified by MS and quantified: 1-penten-3-ol, 1-pentanol, 1-octen-3-ol,

397   pentanal, *t*-2-pentenal, *t*-2-hexenal, hexanal, *t,t*-2,4-heptadienal, 4-heptenal, octanal, nonanal,

398   decanal, 1-penten-3-one, 2-ethylfuran, 2-pentylfuran and benzaldehyde. The identity of these

399 volatiles was confirmed by comparison of retention times with those of external standards. Mainly,

400 all volatile secondary oxidation products in the present study showed similar development patterns

401 in volatile concentrations during the storage period, with a lag phase followed by particularly

402 increase in the later part of the storage period (data not shown for all volatiles).

403 In Figure 3 (B, C and D) three representatives of oxidation products from EPA and DHA are

404 shown, namely 1-penten-3-one, 1-penten-3-ol and t,t-2,4-heptadienal, which have all been

405 recognised as a decomposition products of EPA and DHA (Venkateshwarlu et al., 2004a). In

406 addition, 1-penten-3-one has been suggested as one of the markers for fishy and metallic off-

407 flavours in fish-oil-enriched foods (Venkateshwarlu et al., 2004b). Both the length of lag phase and

408 the amount of volatiles for three representatives differed between samples prepared by primary and

409 secondary emulsions. During storage period, the samples prepared by secondary emulsions

410 produced less amount volatiles and the lag phase was longer compare to samples prepared by

411 primary emulsions.

412 The lag phases of 1-penten-3-one were generally shorter than for 1-penten-3-ol. A shorter lag phase

413 of 1-penten-3-one than 1-penten-3-ol has been observed previously in determination of lipid

414 oxidation in fish oil enriched foods (Let et al., 2003; Sørensen et al., 2012; Hermund et al., 2015).

415 This has been found to the related to the reduction of 1-penten-3-one to 1-penten-3-ol. Between

416 samples added with extracts, both acetone and ethanol extracts produced less amount of volatiles

417 compared to blank samples that was most notable in samples prepared by secondary emulsions

418 through to the end of storage.

419

420 3.5. *Microstructure and lipid distribution*

421 Confocal light microscopy was applied to investigate the lipid distribution in the granola bars to

422 understand how fish oil emulsions were incorporated in the bars prepared with primary and

423   secondary emulsions in the presence of ethanol extract. The starch granules were clearly

424   distinguished in the water phase as green grains with cracks in the middle. These granules were

425   widely distributed in the matrix both as large and smaller grains. According to the images the oil

426   was not highly associated with air. All images showed that oil was not only found as spherical oil

427   droplets but also as large oil pools in the bars, which indicated ruptured droplets. More evenly

428   distributed spherical oil droplets were observed in the bars prepared with secondary emulsion. The

429   final water content of the bars was low and the confocal images (Figure 4) though show that the

430   bars contained few large spherical water droplets that could be a site for lipid oxidation. The

431   confocal images also showed that protein bands were formed (light green areas). These bands

432   surrounded some of the oil droplets forming a physical barrier on the surface of the droplets, which

433   could influence the lipid oxidation. This is typically seen in protein emulsified o/w emulsions.

434

435   *3.6.   Principal component analysis*

436   PCA was carried out to visualise correlations between types of extract used in the granola bars

437   (samples) and the oxidation parameters (variables). PCAs were carried out at three different storage

438   time points (Figure 5 A, B and C).

439

440   In Figure 5A, PCA on data retrieved from week 0 was shown. The first two principal components in

441   this PCA explained 52 and 14% of the total variance in the data set, respectively. In the beginning

442   of storage period, most of the variables were located on the left side of the chart whereas samples

443   Em1A, Em2blnk, Em2E were located on the right side away from the positions of almost all

444   variables. Em1W and Em1blnk have similar responses, whereas Em2W and Em1E were located far

445   on the upper side of the origin, and this indicates that has roughly opposite response patterns.

446   Accounting for PC1, all volatiles has similar effects on samples. Em2A was the first sample hit the

447   vectors of volatiles by its imaginary perpendicular line, indicating that the amount of volatiles at the

448   beginning of storage was highly associated with Em2A and, at the opposite direction where

449   Em2blnk and Em1A was the last samples were less associated with the amount of volatiles. With

450   regards to samples that have rancid smell, also more likely have higher PV scores since the angle

451   between those variables were very small.

452

453   In Figure 5B, the PCA on data from samples of week 5 is depicted. PC1 describes 51% of the

454   model and PC2 22%. At week 5, a few variables located on the left side of graph moved next to the

455   other variables, Em2blnk protected its location still far from all variables. Compare to week 0,

456   Em2A moved its location on the opposite side of the graph and with Em2W and Em2blnk became

457   the samples that have least correlation with the volatiles and other oxidation parameters. Among

458   samples prepared by secondary emulsion, the samples with ethanol extract (Em2E) showed similar

459   responses to samples prepared by primary emulsion. Except sweet smell and taste, other sensory

460   parameters have the same direction along with PV, the samples that have fishy smell seems also

461   more likely have higher PV scores by week 5. Em1blnk has the highest correlation with volatile

462   secondary oxidation products, followed by Em1W and Em1A.

463

464   In Figure 5C, the PCA on data from samples from the last week of storage was shown including the

465   decrease in EPA, DHA and total PUFAs (RDA%). The first two principal components in this PCA

466   explained 45 and 24% of the total variance in the data set, respectively. At the end of storage,

467   similar to the previous graphs for week 5 and 0, Em2A was located on the left side far from

468   oxidation parameters accompanied by Em2E that were fairly dissimilar than Em1E that located on

469   the far right side with regards to the amount of volatiles. Among samples prepared by secondary

470   emulsions, Em2W and Em2blnk showed similar responses. All sensory parameters except sweet

taste, was located on the same zone, very close to PV values. The decrease in the amount of

unsaturated fatty acids was located far from other oxidation parameters and samples associated with

them.

During storage of samples from week 0, generally samples prepared with secondary emulsions

located far from oxidation parameters. At week 10, the far lowest observed content of volatiles and

other oxidation parameters in this study was found for Em2A and Em2E.

From our results, when fish oil added into granola bars by using secondary emulsion system,

oxidative stability during storage at room temperature is improved compared to samples enriched

with primary fish oil emulsion system. In the same emulsion system, acetone and ethanol extracts

provided better oxidative stability than water extracts. The decrease in some n-3 PUFAs were

reduced by the addition of ethanol extracts in the secondary emulsion significantly, and among

other samples there was no significant difference in the decrease of some n-3 PUFAs during the

storage. Compare to samples enriched with primary emulsion, the development of volatile fish oil

oxidation products relate to fish oil oxidation was reduced to a great extent in samples enriched with

secondary emulsion system.

 High antioxidant efficacy of ethanol and acetone extracts from *F. vesiculosus* was also found by

Honold et al (2015) when tested in mayonnaise fortified with 5% FO. It was found that both

extracts could lower the formation of peroxides and increase the lag phase of the formation of some

volatile compounds compared to the blank samples. In the study of Hermund et al. (2015), where

similar *F. vesiculosus* water extract as in the present study were tested in 5% FO enriched milk and

mayonnaise, it was found that the low concentration of water extract in mayonnaise showed no

antioxidant effect towards the formation of peroxides whereas the high concentration showed low

antioxidant activity. In our previous study (Karadağ et al., 2015), where we investigated two

different concentrations of extracts, water extract added in either low or high concentration showed

only no or very weak antioxidant activity and was not as efficient as the ethanol and acetone

extracts.

Wang et al. (2009, 2010 & 2012), Hermund et al. (2015) and Honold et al. (2015) used the same *F.*

vesiculosus extracts in the present study, and they showed the high radical scavenging activity of *F.*

vesiculosus extracts has been related to their high phlorotannin content, the major phenolic

compound in the brown algae and thereby also the total phenolic content (Wang et al., 2009, 2010

and 2012). Furthermore, polyphenols such as phlorotannins from *Fucus* sp. are also good metal

chelators (Smith et al., 1986; Fukumoto & Mazza, 2000).

4. Conclusions

Additional protection against lipid oxidation was obtained when multilayer fish oil emulsions were

added to the granola bars especially in combination with acetonic (AE) and ethanolic (EE) extract.

The results from this study indicated the importance of the presence of high levels of phlorotannins

in the extracts, thus AE and EE can be recommended for application in these types of low moisture

food products, even at the low concentrations tested (0.4 g dw/kg product).

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Table 1. Composition of water (W), ethanol (E) and acetone extract (A), according to Hermund et al. (2015) and Honold et al. (2015)

Composition of extracts	Unit	W	E	A
Total Phenolic Content	g GAE/100 g dw	18.4±0.1	20.4±2.4	23.2±1.1
Protein	% w/w dw	nd	1.7±0.0	2.3±0.0
α- tocopherol		19.0±1.9	2.4±1.9	4.0±0.3
ß- tocopherol	μg/g dw	2.9±0.0	1.7±0.3	1.9±0.7
γ- tocopherol		6.2±0.2	1.8±0.5	2.5±0.8
δ- tocopherol		24.5±1.2	18.0±4.4	12.9±0.6
Iron	μg/g dw	4.4±1.0	14.5±0.1	9.5±1.1
Chlorophylls	μg/mg dw	0.5±0.0	1.9±0.1	0.8±0.1
Carotenoids		4.0±0.5	6.9±1.0	0.7±0.1

W, E, A: Water, Ethanol and Acetone extracts of *F. vesiculosus*
nd: not determined

Table 2. Granola bar formulation (g/100 g)

Ingredient	Quantity
Rolled oats	16.0
Syrup	8.0
Honey	8.0
Wheat flour	11.6
Nut/fig/cocoa spread*	7.0
Rice crispies	7.0
Raisin	7.0
Apricot	7.0
Soft brown sugar	4.2
Maltodextrin	4.2
25% Fish oil emulsion	20

* Hazelnuts (8.6%) were toasted and blended (Kenwood kitchen blender) together with nut/fig mix (57.1%), cocoa powder (5.7%) and water (28.6%).

Table 3. Particle size (μm), zeta potential (mV) and peroxide values (meqO$_2$/kgoil) of primary (1) and secondary (2) fish oil emulsions containing acetone, ethanol and water extract of *F. vesiculosus*. Measurements were done on the day of emulsion preparation

	Particle size, μm (d$_{3,2}$)	Zeta potential (mV)	PV (meqO$_2$/kg oil)
EM1Blnk	0.46±0.05[a]	-25.67±3.32 [a]	0.85±0.00 [a]
EM1A	0.65±0.09[b]	-27.81±2.00 [a]	2.79±0.13 [b]
EM1E	0.63±0.08[b]	-26.04±2.32 [a]	1.76±0.11 [c]
EM1W	0.52±0.07[a]	-26.72±1.88 [a]	1.72±0.24 [c]
EM2Blnk	0.93±0.05[c]	20.37±1.05 [b]	1.10±0.05 [a]
EM2A	1.59±0.08[d]	20.05±1.58 [b]	2.82±0.15 [b]
EM2E	1.37±0.23[f]	20.17±0.93 [b]	2.02±0.41 [c]
EM2W	0.95±0.02[c]	19.62±1.00 [b]	1.63±0.15 c

Values are means±SD of at least 3 parallel measurements
Different letters (a-c) in each column showed significant difference (p<0.05)

Table 4. Dry matter (%w/w), metal content (µg/g bar) and relative decrease in area (RDA) % of EPA, DHA, 18:4(n-3) and PUFAs in the FO-enriched granola bars from week to 10

Sample	Dry matter (%)	Metal content (µg/g bar)		Relative decrease in area%			
		Fe	Cu	EPA	DHA	18:4(n-3)	PUFAs*
EM1Blnk	76.58±1.71	18.91±2.22[a]	2.04±0.20 [a]	-0.61[a]	-0.84[ab]	0.005 [a]	-1.79 [a]
EM1A	81.53±0.66	25.11±0.13[a]	3.74±1.37 [a]	-0.57[a]	-0.75 [ab]	-0.01 [a]	-1.99 [a]
EM1E	78.32±3.69	21.08±0.85[a]	2.32±0.03 [a]	-0.50 [a]	-1.14 [a]	-0.04 [a]	-1.93 [a]
EM1W	80.01±1.69	20.42±0.66[a]	2.68±0.41 [a]	-0.80 [a]	-1.10 [a]	0.01 [a]	-1.94 [a]
EM2Blnk	75.32±0.35	22.08±0.92[a]	3.22±0.12 [a]	-1.07 [a]	-1.40 [a]	-0.1 [a]	-2.49 [a]
EM2A	78.69±2.47	20.82±1.19[a]	2.18±0.05 [a]	-0.74 [a]	-0.74 [ab]	0.01 [a]	-2.89 [a]
EM2E	75.55±2.68	22.02±1.47[a]	2.85±0.12 [a]	0.28[b]	0.19 [b]	0.56 [b]	1,18[b]
EM2W	76.61±1.70	20.44±4.32[a]	2.18±0.32 [a]	-0.44[a]	-0.46 [ab]	0.08 [a]	-1.34 [a]

a, b: significance between samples within the same column
*PUFAs: total of C18:2 (n-6), C18:3 (n-3), C18:4 (n-3), C20:4 (n-3), C20:5 (n-3, EPA) and C22:6 (n-3, DHA)
RDA= Week 10- Week 0
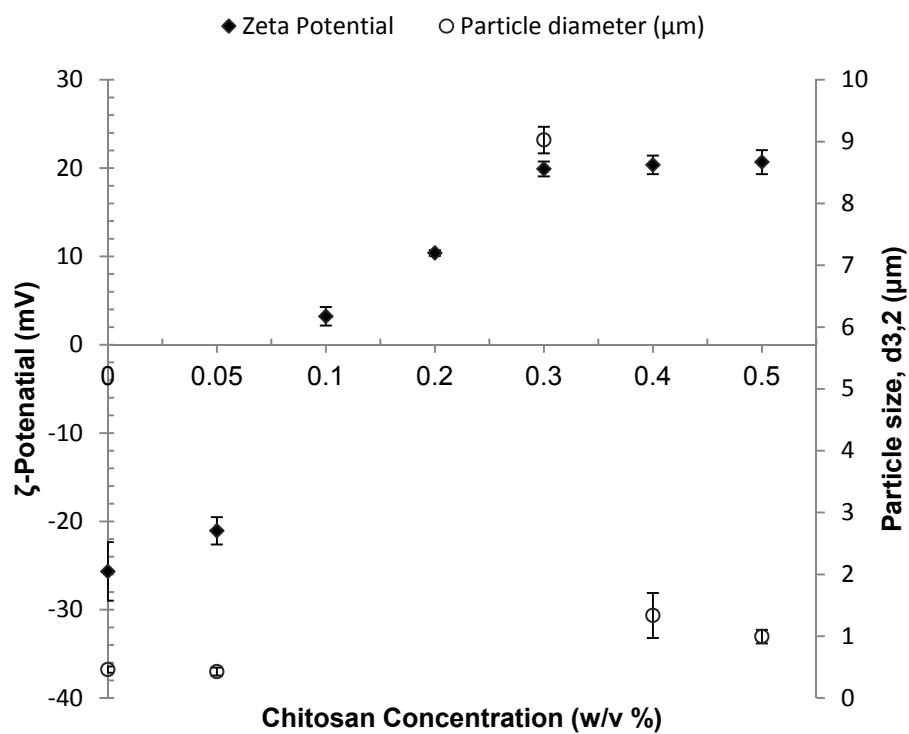
Figure 1. Change in ζ-potential and particle size of emulsion after addition of 0 to 0.5, w/v% chitosan to primary emulsion stabilized by Na-cas (25 % oil, 0.6 % Na-cas and chitosan)
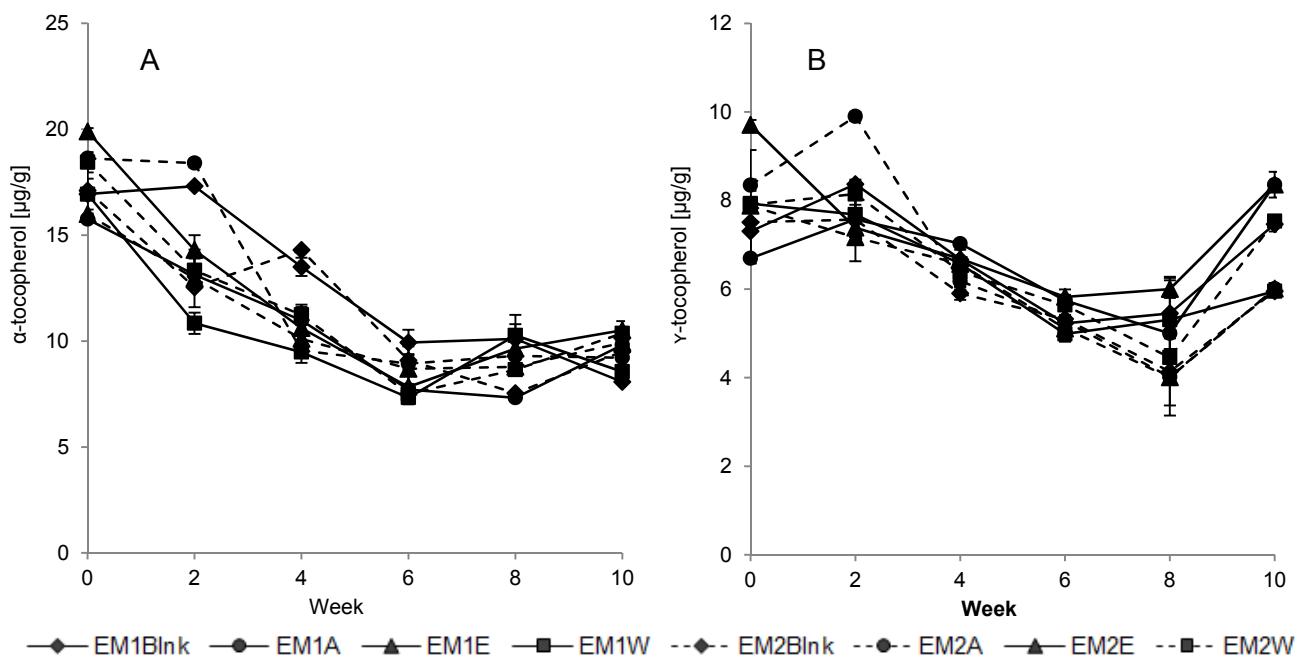
Figure 2. Change in two tocopherol homologues (α and γ-tocopherol) (μg/g), in granola bars stored dark for 10 weeks at room temperature (n=2). The bars were enriched fish oil emulsions (EM1: primary emulsion; EM2: secondary emulsions) containing acetone, ethanol and water extracts of *F. vesiculosus* and blank (without extract)
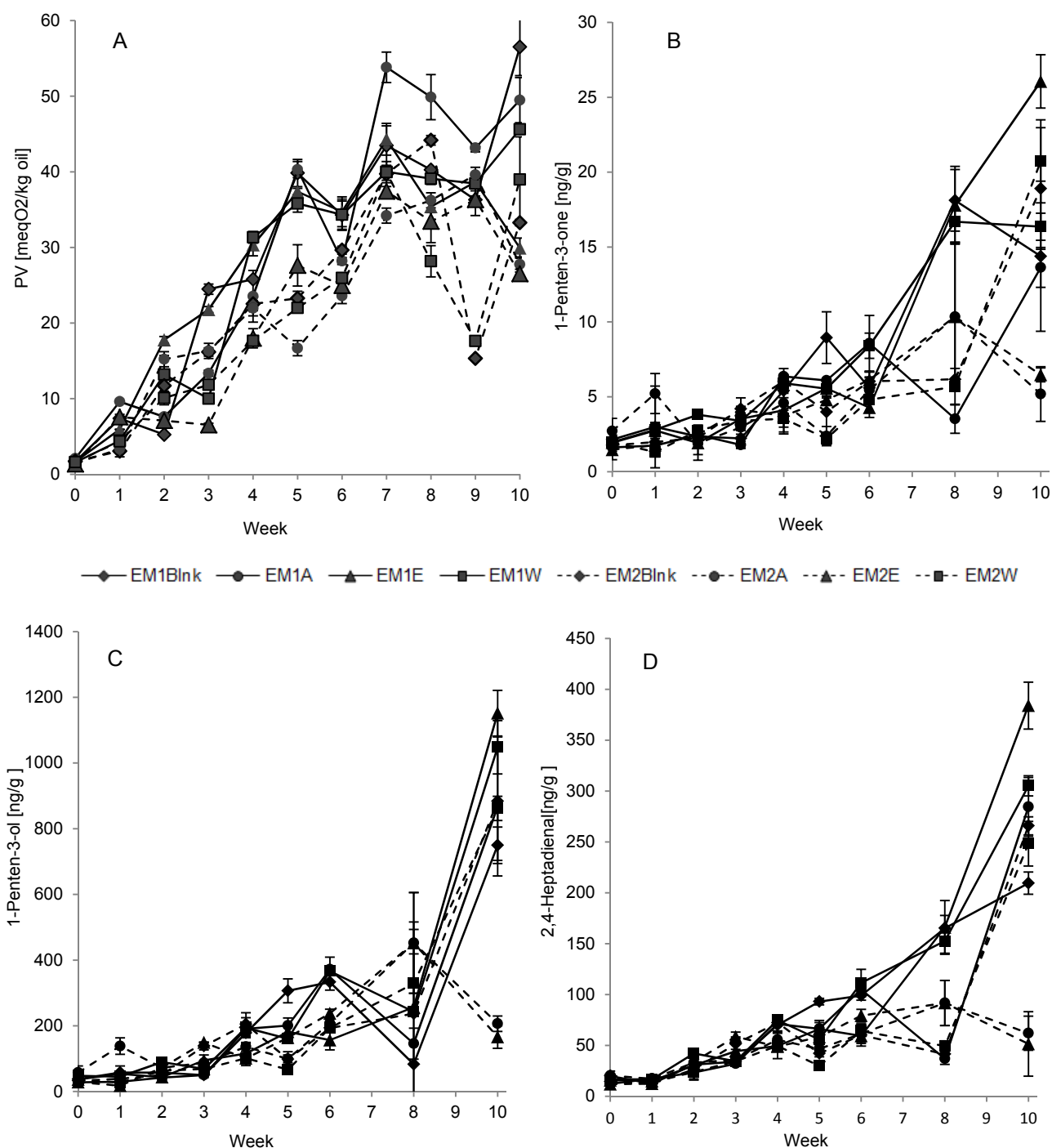
Figure 3. Peroxide values (meq/kg oil) (A) and development of volatile secondary oxidation products (ng/g granola bar) (B-D) in granola bars enriched with fish oil emulsions (EM1: primary emulsion; EM2: secondary emulsions) containing acetone, ethanol and water extract of *F. vesiculosus*, blank (without extract) and stored dark for 10 weeks at room temperature. The development of volatile secondary oxidation products exemplified by 1-penten-3-one (B), 1-penten-3-ol (C), and *t,t*-2,4-heptadienal (D). Error bars indicate SD of the measurements (n = 2 for PV and n = 3 for volatiles compounds).

Figure 4. CLSM images of granola bars prepared by primary (A) and secondary (B) fish oil emulsions in the presence of ethanol extract
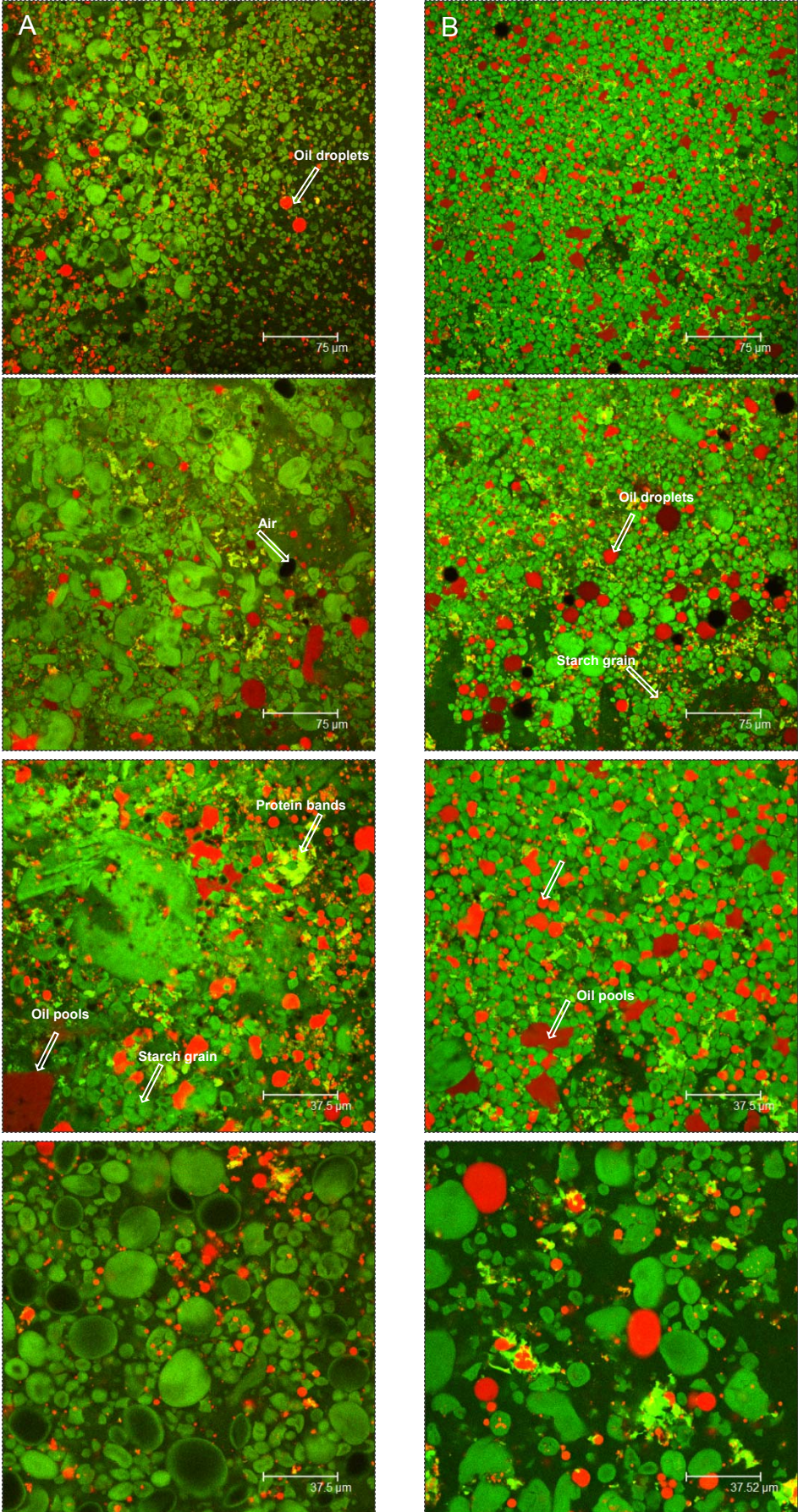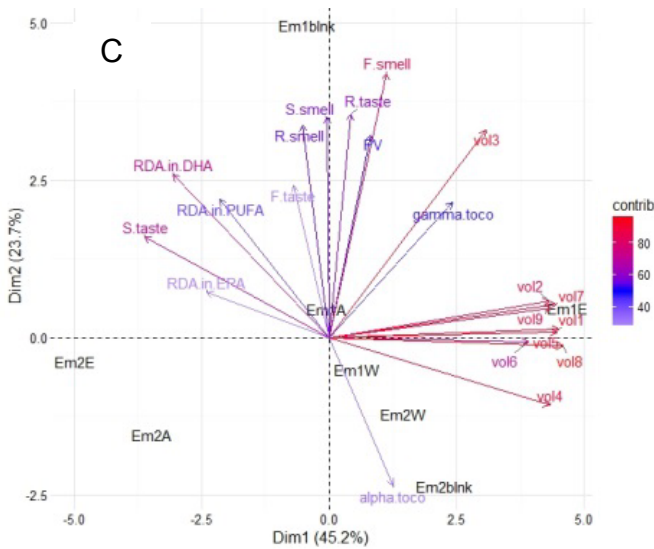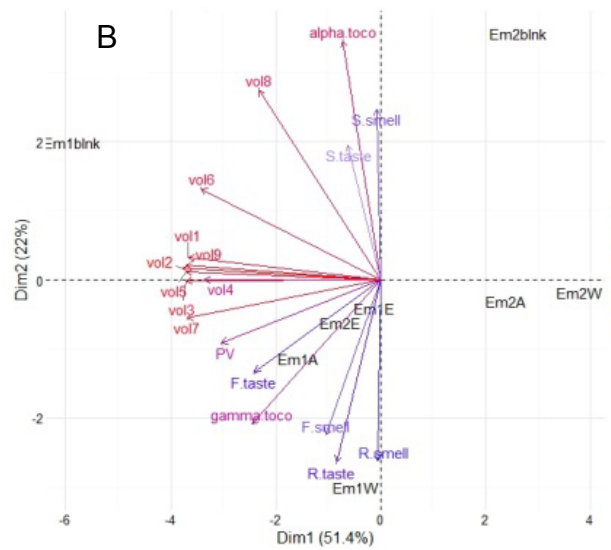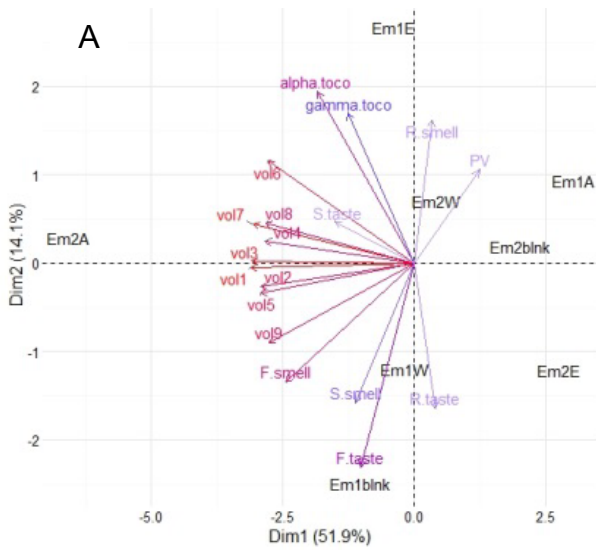
Figure 5. A, B and C are bi-plots with the samples on week 0(A), 5 (4 for tocopherols, B) and 10 (C) for samples Em1blnk, Em1A, Em1E, Em1W, Em2blnk, Em2A, Em2E, Em2W and variables: PV (peroxide value), vol1 (1-penten-3-one), vol2 (1-penten-3-ol), vol3 (1-pentanol), vol4 (4-heptenal), vol5 (2-hexenal), vol6 (hexenal), vol7 (1-octen-3-ol), vol8 (octanal), vol9 (2,4-heptadienal), alpha.toco, gamma.toco, Fishy.smell, Rancis.smell, Fishy.taste, and Rancid.taste. For week 10 reductions in area% of EPA (RDAinEPA), DHA(RDAinDHA) and PUFAs (RDAinPUFA) are included as well

PAPER V

Antioxidant effect of aqueous and acetonic extracts of *Fucus vesiculosus* on oxidative stability of cosmetic emulsions

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Antioxidant effect of water and acetone extracts of Fucus vesiculosus on oxidative stability of skin care emulsions

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Antioxidant effect of water and acetone extracts of *Fucus vesiculosus* on oxidative stability of skin care emulsions

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Key words: cosmetic emulsion, brown algae, lipid oxidation, skin care emulsion

Abbreviations: RF, reference; WE, water extract; AE, acetone extract; TTC, total tocopherol content; PV, peroxide value; VC, volatile compounds;

1 **ABSTRACT**

2 A water and an acetone extract of the Icelandic brown algae *Fucus vesiculosus* were

3 evaluated as potential natural sources of antioxidant compounds in skin care emulsions.

4 To assess their efficacy in inhibiting lipid oxidation caused by photo- or thermoxidation,

5 they were stored in darkness and room temperature as control conditions, and compared

6 to samples stored under accelerated conditions (light and room temperature, or darkness

7 and 40 ℃). The presence of extracts in the skin care emulsions induced remarkable

8 colour changes when the emulsions were exposed to light, and more extensively under

9 high temperature. High temperature also caused greater increments in the droplet size of

10 the emulsions. The analysis of the tocopherol content, peroxide value and volatile

11 compounds during the storage revealed that, whereas both water and acetone extracts

12 showed (at 2 mg/g of emulsion) protective effect against thermooxidation, only the

13 water extract showed antioxidant activity against photooxidation.

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1. INTRODUCTION

15

16   Natural derived ingredients combined with carrier agents, preservatives, surfactants,

17   humectants and emulsifiers are commonly used in skin care products. A natural

18   ingredient is based on botanically sourced ingredients currently existing in nature (such

19   as herbs, roots, essential oils and flowers), in order to reduce synthetic compounds in

20   the final product. Nowadays, there is an increasing interest in natural ingredients [1]

21   because of the negative perception of the synthetic ones. Thus, the evolution of the

22   cosmetic industry to adapt products to the trends of the XXI century consumer has

23   given rise to new challenges.

24   Emulsions are the most common type of delivery system used in cosmetics, with creams

25   and lotions being the best-known. Skin care emulsions enable a wide variety of active

26   ingredients to be quickly delivered to skin. In this sense, there are many factors that can

27   potentially influence the physical and oxidative stability of these emulsions, such as

28   fatty acids and ionic composition, type and concentration of antioxidants and

29   prooxidants, emulsion droplet size and interfacial properties [2, 3, 4, 5].

30   Lipid oxidation can occur in skin care emulsions [6, 7] and can be triggered or enhanced

31   by light and/or high temperatures. Moreover, the high content of vegetable oils, e.g.

32   almond oil, in skin care emulsion formulations might contribute to induction of lipid

33   oxidation, causing unpleasant odours, colour changes and in consequence, low quality

34   products [6, 7].

35   Therefore, it is important to limit lipid oxidation and to extend the shelf life of skin care

36   products using natural antioxidants. In addition, some natural antioxidants can give the

37   skin product added functional value. It has recently been suggested that the use of

38   natural antioxidants, such as vitamins A and E, in skin care formulations could provide

39   a preventive therapy for skin photoaging [8, 9]. Moreover, beauty-improving

40   formulations of skin care emulsion with seaweed extracts or micro algae added have

41   also been reported [10].

42   Vitamin E is one of the most used natural antioxidants in skin care products, usually

43   added due to its radical scavenging activity [9]. However, in highly complex matrices

44   containing trace metals, such as cosmetics products [11], other antioxidant properties

45   such as metal chelating ability might be of relevance. Therefore, to stabilise lipid rich

46   skin care products, extra addition of antioxidants might be necessary.

47   Natural derived antioxidants from various plants and marine algae have shown great

48   potential in improving oxidative stability in these kinds of products. A high variety of

49   bioactive compounds, such as pigments, sulphated polysaccharides, proteins and

50   polyphenols, have been described for different types of Danish brown and red algae by

51   Farvin and Jacobsen [12]. Especially, the high content of phlorotannins, the major

52   polyphenolic compounds in brown algae, has been related to high antioxidant activity,

53   as these compounds can work both as radical scavengers and metal chelators [13, 14].

54   Furthermore, phlorotannins have been shown to possess biological activity of potential

55   medicinal value making them valuable in development of nutraceutical, pharmaceutical

56   and cosmetic products [15, 16]. Balboa et al. [1] successfully used a *Sargassum*

57   *muticum* extract to improve the oxidative stability of oil-in-water model emulsions with

58   cosmetic purposes. Farvin and Jacobsen [12] found that, compared to other Danish

59   brown alga species, *Fucus vesiculosus* had higher phenolic content and exhibited the

60   highest antioxidant activity *in vitro*. Wang et al. [14, 17] found that the high *in vitro*

61   antioxidant activity of extracts derived from the Icelandic *F. vesiculosus* were related to

62   a high phenolic content and identified the phlorotannin tetramer, fucodiphloroethol E, to

63   be the main contributor to this activity. Moreover, Hermund et al. [18] and Karadağ et

64   al. [19] studied the application of Icelandic *F. vesiculosus* extract as potential

65   antioxidant against lipid oxidation in fish-oil-enriched food products, i.e. food

66   emulsions such as mayonnaise, milk and granola bars added preemulsified fish oil, and

67   found promising results.

68   Whereas the *in vitro* antioxidant properties of *F. vesiculosus* have been widely studied

69   [14, 20], applied studies on the antioxidant activity of *F. vesiculosus* extracts to hinder

70   lipid oxidation are sparse [18, 19, 21, 22].

71   The aim of this study was to evaluate the antioxidant properties of two extracts obtained

72   from Icelandic brown algae *F. vesiculosus* (water and acetone extract) in terms of

73   assessing their efficacy to inhibit lipid oxidation during the storage of skin care

74   emulsions, at room temperature in darkness and under two different accelerated

75   conditions (photo- and thermooxidation).

76

77 **2. MATERIAL AND METHODS**

78 **2.1. Materials**

79 The ingredients for the formulation of the skin care emulsion were purchased from

80 Urtegaarden (Allingåbro, Denmark). All solvents used were of high-performance liquid

81 chromatography (HPLC) grade and purchased from Lab-Scan (Dublin, Ireland).

82 External standards were purchased from Sigma Aldrich (Steinheim, Germany).

83

84 **2.2. Extraction**

85 The two extracts used in this study were provided by Matís in Iceland and have been

86 used in previous studies of foods (water extract previously used by Hermund et al. [18],

87 the acetone extract by Honold et al. [22] and both extract by Karadağ et al. [19]).

88 The extractions were carried out according to Wang et al. [14, 17]. The seaweed was

89 collected in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in

90 2011. At the collecting spot the seaweed was washed with clean seawater to remove

91 salt, epiphytes and sand attached to the surfaces of the samples and transported to the

92 laboratory. The samples were rinsed with tap water and wiped with paper towel. The

93 samples were freeze-dried, pulverised into powder and stored at -80 °C prior to

94 extraction.

95 The extracts were produced as follows: Five grams of the algal powder was mixed with

96 100 mL of distilled water or 70 % aqueous acetone (v/v). Hereafter these were

97 incubated on a platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison,

98 NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2168

99 g for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. Each extraction was

100 conducted in duplicate. The extracts were stored at -80 °C until use.

101   The water extract (WE) composition was as follows: phenolic content, 14.73 g gallic

102   acid equivalent/100 g extract; chlorophylls, 0.46 μg/mg extract; xanthophylls, 2.17

103   μg/mg extract; carotenes, 1.72 μg/mg extract; iron, 4.39 μg/mg extract and copper, 0.91

104   μg/mg extract. The acetone extract (AE) composition was as follows: phenolic content,

105   18.55 g gallic acid equivalent/100 g extract; chlorophylls, 0.85 μg/mg extract;

106   xanthophylls, 0.75 μg/mg extract; iron, 9.53 μg/mg extract and copper, 1.21 μg/mg

107   extract.

108

109   **2.3. Skin care emulsion production and storage conditions**

110   The two *F. vesiculosus* extracts, WE and AE were applied to the skin care emulsion in

111   two concentrations, 1 and 2 mg/g of skin care emulsion (concentration 1 and 2,

112   respectively). The concentrations have previous shown to increase the stability of fish-

113   oil-enriched foods [18]. Thus, five different types of skin care emulsions were finally

114   obtained: RF (reference, without extract), WE1, WE2, AE1, AE2. Table 1 shows all the

115   ingredients for the water phase and the oil phase (including the extract).

116

117   Table 1. Formulation of the different samples.

Water phase	
Demineralized water	52.5 g/ 100 g
Aloe vera water	10.0 g/ 100 g
Glycerin	6.3 g/ 100 g
MF fat	3.6 g/ 100 g
Natriumbenzoat	0.6 g/ 100 g
F. vesiculosus extract (water or acetonic)	1 or 2 mg/g
Oil phase	
Almond oil	21.8 g/ 100 g
Lanette wax	2.0 g/ 100 g
VE fat	1.8 g/ 100 g
Vitamin E	0.9 g/ 100 g

118

119   The ingredients were weighted in individual pots and heated to 70-75 °C. The oily

120   phase was slowly poured into the water phase under powerful steering (9.500 rpm,

121   Ultra-Turrax® T25basic). After the homogenization process, the emulsions were cooled

122   to room temperature. The skin care emulsions were packed in transparent 50 ml

123   containers. Then the samples were stored under three different conditions: room

124   temperature (21.2±0.7°C) and darkness (A0), room temperature (24.4±0.3°C) and light

125   (A+) and high temperature (42.3±1.5°C) and darkness (H0). The samples were analysed

126   at different storage times (0, 7, 21, 35 and 56 days).

127

128   **2.4. Lipid extraction**

129   Lipids were extracted from the skin care emulsions according to the method described

130   by Iverson, Lang, and Cooper [23] based on the method of Bligh and Dyer [24]. For

131   each sample, two oil extractions were performed and analyzed independently. Resulting

132   lipid extracts were used as starting material for the analysis of peroxides, fatty acid

133   composition and tocopherol content.

134

135   **2.5. Fatty acid composition (fatty acid methyl esters, FAME)**

136   The fatty acid composition of the oil phases was determined after fatty acid methylation

137   and analysis by GC-FID. The Bligh and Dyer [24] lipid extract from skin care emulsion,

138   corresponding to 30-60 mg lipid, were weighted in vials. 100 µL toluene, 200 µL

139   heptane with 0.01 % (v/v) BHT and 100 µL internal standard (C23:0) (2 % w/v) were

140   added. One mL of BF_3 in methanol was added to the lipid extract mixture and the lipids

141   were methylated in a one-step procedure using a microwave oven (Multiwave3000

142   SOLV, Anton Paar, Graz, Austria) with a 64MG5 rotor. The settings for the microwave

143   were 5 min at 500 Watt followed by 10 min cooling. The fatty acid methyl esters

144   (FAMEs) were washed with 1 mL saturated NaCl and 0.7 mL heptane with 0.01 % (v/v)

145   BHT. The heptane phase was transferred to a GC vial and FAMEs were analysed by GC

146   (HP 5890A, Agilent Technologies, Palo Alto, CA, USA) according to AOCS [25]. For

147   separation DB127-7012 column (10 m x ID 0.1 mm x 0.1 μm film thickness, Agilent

148   Technologies, Palo Alto, CA, USA) was used. Injection volume was 0.2 μL in split

149   mode (1:50). The initial temperature of the GC-oven was 160°C. The temperature was

150   set to increase gradually being as follows: 160-200°C (10.6°C/min), 200°C kept for 0.3

151   min, 200-220°C (10.6°C/ min), 220°C kept for 1 min, 220-240°C (10.6°C/min) and kept

152   at 240°C for 3.8 min. The measurements were performed at storage day 0 and 56, in

153   duplicates, and the results were given in % of total area.

154   **2.6. Tocopherol content**

155   The lipid extracts from the skin care emulsions were evaporated under nitrogen and

156   dissolved in heptane. The samples were analysed by HPLC (Agilent 1100 Series,

157   Agilent Technology) according to AOCS [25] to quantify the contents of α-, β-, γ- and

158   δ-tocopherols. These tocopherol homologues were separated using a silica column

159   (Waters, Dublin, Ireland, 150 mm, 4.6 mm, 3 μm silica film). A stock solution added 10

160   mg tocopherols (mixture of α-, β-, γ- and δ-tocopherols) per litre was prepared to

161   determine the retention time of the tocopherols and the peak areas of the given

162   standards. The peak areas of the standard solution were used to calculate the tocopherol

163   content of the samples. The analyses were done in duplicates and results were reported

164   as μg tocopherol/g skin care emulsion.

165

166   **2.7. Peroxide value (PV)**

167   PVs of the lipid extract of the skin care emulsions were determined at all sampling

168   points. This was done according to the method by Shantha and Decker [26], based on

169   the formation of an iron-thiocyanate complex. The coloured complex was measured

170   spectrophotometrically at 500 nm (Shimadzu UV1800, Shimadzu Scientific

171   Instruments, Columbia, MD, USA). The analyses were done in duplicate and the results

172   were expressed in milliequivalents peroxides per kg oil (meq O_2/kg oil). In addition, for

173   every sample, oxidation rates were calculated as follows:

174    $$Oxidation\ rate\ (\%) = \frac{\left(PV_{day35\,or\,56} - PV_{day1}\right)}{PV_{day1}} \times 100$$

175

2.8. Volatile compounds (VC)

177   Tenax GR™ packed tubes were used to collect volatile compounds by dynamic

178   headspace. The collection of the volatile compounds was carried out using 4 g of

179   emulsion (including 30 mg internal standard (30 µg/g of 4-methyl-1-pentanol in

180   ethanol)) and 20 mL of distilled water. The volatile secondary oxidation products were

181   collected at 45 °C under purging with nitrogen (flow of 150 mL/min) for 30 min,

182   followed by flushing the Tenax GR™ packed tube with nitrogen (flow of 50 mL/min

183   for 5 min) to remove water. The trapped volatiles were desorbed using an automatic

184   thermal desorber (ATD-400, Perkin- Elmer, Norwalk, CT) connected to an Agilent

185   5890 IIA model gas chromatograph equipped with a HP 5972 mass selective detector.

186   The settings for the MS were: electron ionization mode, 70 eV, mass to charge ratio

187   (*m/z*) scan between 30 and 250 mAU. Chromatographic separation of volatile

188   compounds was performed on a DB1701 column (30m × ID 0.25mm × 0.5 µm film

189   thickness, J&W Scientific, Folsom, CA, USA) using helium gas flow (1.3 mL/min).

190   The temperature programme was as follows: 3 min at 35°C, 3°C/min from 35 to 120°C,

191   7°C/min to 120-160°C, 15°C/min 160-200°C and hold for 4 min at 200°C.

192   The auto sampler collector setting details were: 9.2 psi, outlet split: 5.0 mL/min,

193   desorption flow: 60 mL/min. The analysis was performed in triplicate in all sampling

194   points and the results were given in ng/g of emulsion.

195   The quantification of the different volatiles was done by the use of a calibration curve

196   prepared from the following external standards dissolved in ethanol: pentanal

197   (calibration range, c.r: 0.007-3.77 mg/g), hexanal (c.r: 0.005-2.69 mg/g), heptanal (c.r:

198   0.008-4.15 mg/g), *trans*-2-heptenal (c.r: 0.005-2.95 mg/g), octanal (c.r.0.006-3.11

199   mg/g), *trans*-2-octenal (c.r: 0.005-2.91), 1-octen-3-ol (c.r: 0.006-3.01 mg/g) and 2-

200   ethyl-1-hexanol (c.r: 0.006-3.19 mg/g). 1 μL of every solution prepared at different

201   concentrations, was added to a Tenax GR™ tube and flushed with nitrogen (flow of 50

202   mL/min for 5 min) to remove the solvent. Then, the volatiles were analyzed in the same

203   way as for the samples. Results for each compound were expressed as ng/g of extract,

204   and oxidation rates were calculated as follows:

205   $$Oxidation\ rate\ (\%) = \frac{\left(VC_{day35or56} - VC_{day1}\right)}{VC_{day1}} \times 100$$

206

207   2.9. Droplet size distribution

208   The size of fat globules in the o/w emulsion systems was determined by laser diffraction

209   using a Mastersizer 2000 (Malvern Ins.,Worcestershire, UK).

210   The skin care emulsion was diluted 1:9 in SDS buffer (10 mM NaH_2PO_4, 5 mM SDS,

211   pH 7) prior to analysis. Droplets of the diluted skin care emulsion was added to

212   recirculation water (3000 rpm) reaching an obscuration of 12–14%. The set-up used was

213   the Fraunhofer method, which assumed that all sizes of particles scatter light with the

214   same efficiency and that the particles are opaque and transmits no light. The refractive

215   index (RI) of sunflower oil at 1.469 and water at 1.330 were used as particle and

216   dispersant, respectively. Measurements were performed on day 0 and 56, in triplicates.

217 Results were given as surface area mean diameter D(0.9), which indicates that 90% of

218 the volume of the oil droplets is smaller than this value.

219

220 **2.10. Colour determination**

221 Colour of skin care emulsions was measured using a digital colorimeter (Chromameter-

222 2 CR-200, Minolta, Osaka, Japan) to obtain the colour coordinates L*, a* and b*. These

223 values were used to calculate the euclidean distance value

224 $(\Delta E = \sqrt{\left(L_2^* - L_1^*\right)^2 + \left(a_2^* - a_1^*\right)^2 + \left(b_2^* - b_1^*\right)^2}$) that allowed two types of comparisons:

225 comparison of samples with and without extracts, and also comparison of the initial

226 colour of every sample to that detected along the storage. The measurements were

227 performed in triplicates.

228

229 **2.11. Statistical processing**

230 Mean and standard deviation of results obtained were calculated. For each parameter,

231 one way ANOVA with Tukey-b post hoc multiple comparisons was used in order to

232 evaluate the significant differences among samples and treatments. Within each type of

233 sample, the differences between 0 and 56 days were evaluated by Student t-test.

234 The statistical analysis of data was done using the SPSS 15.0 program (SPSS, INC.,

235 Chicago, IL, USA). Significance level of $p \leq 0.05$ was used for all evaluations.

236

237 **3. RESULTS AND DISCUSSION**

238 **3.1. Physical changes**

239 *3.1.1. Droplet size determination*

240 The distribution of oil droplets in the skin care emulsions was determined at the

241 beginning and the end of storage (Fig. 1).
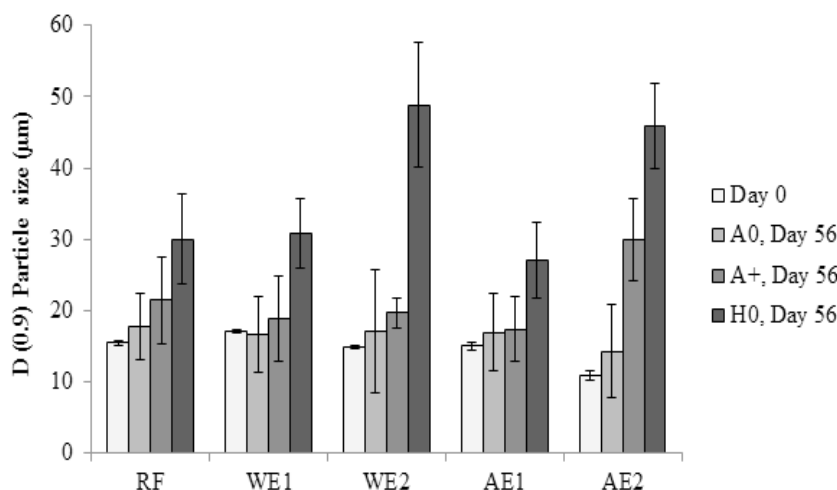
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243 Fig. 1. Droplet size distribution on cosmetic emulsions with or without *F. vesiculosus*

244 extract (water or acetonic) after 56 days of storage under accelerated conditions or at

245 room temperature and dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water

246 extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); A0,

247 darkness and room temperature; A+, light and room temperature; H0, darkness and high

248 temperature).

249

250 D(0.9) value was selected to highlight the differences among samples and treatments.

251 This value indicates that 90% of the volume of droplets is smaller than this value. When

252 an increment in this parameter was observed, a destabilization of the emulsion system

253 had occurred. Regarding the accelerated storage conditions, D(0.9) tended to increase in

254 the presence of light (A+), but a significant increase in D(0.9) was only observed at high

255 temperature (H0). Hence, in all emulsions stored at high temperature, this increased

256   D(0.9) caused a large destabilization of the emulsions, leading to an evident syneresis at

257   the end of the storage (visual evaluation). Due to this observed syneresis effect in H0

258   stored samples at day 56, only samples stored up to 35 days were considered for further

259   analysis in this case. Moreover, WE2 and AE2 showed the highest D(0.9) increments,

260   highlighting that the presence of high doses of these extracts in the skin care emulsions

261   under high temperature, could influence the physical stability of the emulsions. In the

262   case of A+ stored samples, only the addition of 2 mg/g of AE seemed to decrease the

263   stability of the skin care emulsions, although no syneresis was observed.

264   *3.1.2. Colour*

265   In order to evaluate the influence of the presence of some pigments (carotenoids,

266   xantophylls and chlorophylls) on the colour of the emulsions, euclidean distance value

267   (ΔE) was calculated before the storage (day 0). Thus, when comparing colour between

268   the emulsions containing extract and the RF emulsion at day 0, the calculated ΔE were

269   3.90, 6.80, 4.93 and 7.59 for WE1, WE2, AE1, and AE2, respectively. All these values

270   were higher than 2, leading to conclude that clear colour differences were noticed

271   between the samples containing extract and the RF [27], with a strong influence of the

272   concentration and type of *F. vesiculosus* extract applied. The instrumental colour data

273   confirmed that, whereas no differences in lightness (L*) and redness (a*) were found,

274   yellowness (b*) was significantly higher ($p < 0.05$) in the samples containing extract

275   compared to the RF. These colour differences were dose dependent and higher in the

276   AE containing emulsions as compared to WE ones. These colour differences pointed

277   out that the use of seaweed extracts did not perfectly mimic the characteristics of

278   conventional skin care emulsions, due to the presence of pigments.

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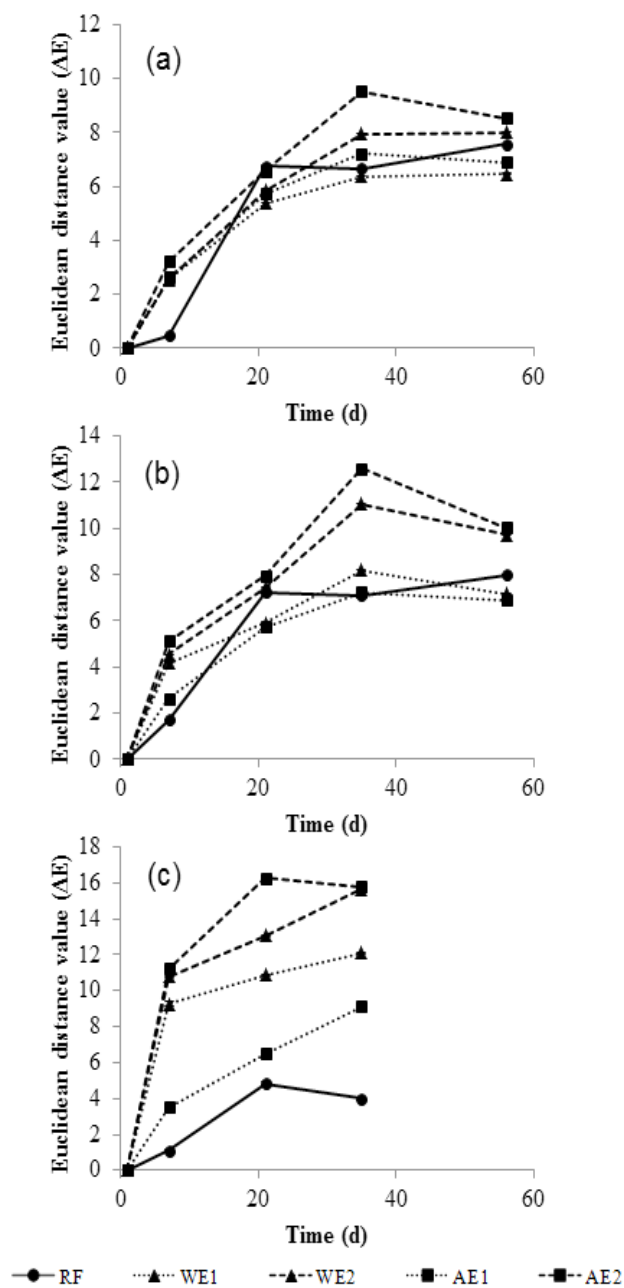
44   280   Fig. 2. Euclidean distance value of the cosmetic emulsions calculated along the storage.

46   281   It compares color at each time of storage to its color at day 0. (a) A0, room temperature

48   282   and darkness; (b) A+, room temperature and light; (c) H0, high temperature and

50   283   darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g);

53   284   AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of

55   285   the measurements.

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286 Additionally, the evolution of the colour was also measured during the storage

287 conditions, and ΔE were also determined (Fig. 2), comparing, in this case, each sample

288 with their own colour at day 0. Results pointed out that the five emulsions did not

289 maintain the colour during storage, as observed by the ΔE increments in all cases. The

290 increment was higher in the samples with higher amount of extract (WE2 and AE2).

291 Particularly light, and mainly temperature (40°C), induced remarkable colour changes

292 in the samples containing extracts, whereas the lowest colour changes in the RF sample

293 at high temperature was observed. These changes were a consequence of an increment

294 in a* value and a reduction in the L* value (data not shown), as the samples became

295 more brown over time. The storage conditions could induce oxidative reactions that

296 might affect pigments such as fucuxanthin and chlorophylls and produce colour changes

297 in the samples. However, this deserves more research.

298

299 **3.2. Oxidative changes**

300 Almond oil (*Prunus amygdalus*) is one of the most valuable skin care oils due to its

301 penetrating, moisturising and restructuring properties, and high content of unsaturated

302 fatty acids. It can be used for numerous skin problems because of their anti-

303 inflammatory, emollient, sclerosant and cicatrizing effects [28]. Therefore, in the

304 present study, almond oil was used in the formulation of the oil-in-water skin care

305 emulsions. However, the susceptibility of the unsaturated lipids present in almond oil to

306 oxidation might be a major cause of quality deterioration and rancidity in the skin care

307 emulsion. The lipid profile of the samples was determined at the beginning and at the

308 end of the storage in every sample, and it was observed that it remained unchanged

309 during the storage period (data not shown), with oleic acid as the major fatty acid,

310 followed by linoleic, and the saturated ones, palmitic and stearic acid. On average, the

311 fatty acid composition was as follows: 14:0, 0.27%; 16:0, 12.0%; 16:1 (ω-7), 0.11%;

312 18:0, 12.1%; 18:1 (ω-9), 53.8%; 18:1 (ω-7), 0.66%; 18:2 (ω-6), 18.4%; 18:3 (ω-3),

313 0.08%; 20:0, 0.28%; 20:1 (ω-11), 0.47%; 20:4 (ω-6), 0.31%; 22:1 (ω-9), 0.17%.

314 *3.2.1. Tocopherol content during storage*

315 Four tocopherol homologues were detected in the skin care emulsions, α-, β-, γ-, δ-

316 tocopherol. The most abundant one at the beginning of the storage was gamma-

317 tocopherol (4895±151 µg tocopherol/g skin care emulsion) followed by delta- (1657±94

318 µg tocopherol/g skin care emulsion), alpha- (1329±72 µg tocopherol/g skin care

319 emulsion) and beta-tocopherol (116±10 µg tocopherol/g skin care emulsion),

320 respectively. The addition of seaweed extract did not affect the content of tocopherols.

321 Similar changes were observed in the four homologues, so the sum of all of them was

322 calculated, and represented as the total tocopherol content (TTC) along the storage (Fig

323 3). The TTC decreased in all samples during storage, and the highest rate of decrease

324 was observed at the high temperature conditions (H0).

325

Fig. 3. Total tocopherol content (µg tocopherol/g cosmetic emulsion) in emulsions with

WE or AE including a control without any extract during storage. (a) A0, room

temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature

and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2

mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars

indicate SD of the measurements.

332   It is worthy to highlight, that the AE showed the highest tocopherol protective effect at

333   all storage conditions, with AE2 being the best concentration. However, WE showed

334   protective effects only when exposed to light (up to 50 days for WE1) and high

335   temperature storage conditions (up to 20 and 35 days for WE1 and WE2, respectively).

336   This protective effect of WE and AE on tocopherols could be due to a synergistic effect

337   between tocopherol and phenolic compounds or pigments, contributing to the

338   regeneration of tocopherol in skin care emulsions containing extracts.

339   *3.2.2. Peroxide value (PV)*

340   The autoxidation of unsaturated fatty acids is a autocatalysed chain reaction through

341   free radical intermediates, and can be accelerated during storage by exposure to light,

342   temperature and in presence of redox metals. On that basis, the primary oxidation

343   compounds, expressed as the peroxide content of the skin care emulsions stored in the

344   different conditions, were determined (Table 2).

345

346 Table 2. Effect of adding water or acetonic *F. vesiculosus* extract on PV (meq O_2/kg oil)

347 of cosmetic emulsions under accelerated stored conditions or room temperature and

348 dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1,

349 acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); S.C, storage condition; A0,

350 darkness and room temperature; A+, light and room temperature; H0, darkness and 40

351 °C.)

		Peroxide value (meq O_2/kg oil)				
	Day 0	S.C	Day 7	Day 21	Day 35	Day 56
RF	5.52±0.51^A	A0	6.85±0.29^Ba	7.31±1.00^Aa	9.48±0.45^Aa	11.62±0.31^A
		A+	8.22±0.52^Ab	11.71±1.07^Bb	13.11±1.19^ABb	18.93±0.63^B***
		H0	11.49±0.84^ABc	11.65±0.29^Ab	15.01±0.78^ABb	
WE1	5.19±0.29^A	A0	6.09±0.04^ABa	6.97±0.45^Aa	8.53±0.36^Aa	10.77±1.25^A
		A+	8.11±0.05^Ab	10.58±0.59^ABb	12.37±0.70^ABb	15.67±1.29^A**
		H0	10.11±1.11^Ac	13.08±1.33^Ac	14.31±0.07^Ac	
WE2	6.58±0.69^B	A0	12.89±0.67^Da	11.95±0.22^Ba	13.96±0.32^Ca	17.67±1.38^C
		A+	14.08±0.21^Da	12.40±0.69^Ba	14.38±1.28^Ba	16.42±1.42^A ns
		H0	13.05±1.60^BCa	11.38±0.19^Aa	13.97±0.19^Aa	
AE1	6.28±0.65^B	A0	14.26±0.29^Ea	13.36±1.03^Bb	15.34±0.35^Db	18.78±0.71^C
		A+	13.16±0.32^Ca	10.90±1.15^ABa	10.34±0.52^Aa	23.97±0.49^C***
		H0	13.73±1.71^Ca	16.58±1.61^Bc	16.47±1.73^Bb	
AE2	6.63±0.75^B	A0	11.31±0.28^Cb	17.03±0.47^Cb	12.36±0.87^Ba	15.14±0.95^B
		A+	9.58±0.23^Ba	9.43±0.35^Aa	17.36±1.99^Cb	18.89±0.55^B**
		H0	10.94±0.21^ABb	10.43±0.78^Aa	14.05±1.05^ABab	

352 *Different capital letters in the same column denote significant differences between*
353 *samples for each storage condition (p<0.05)*
354 *Different small letters in the same column denote significant differences among storage*
355 *conditions for each sample (p<0.05)*
356 *Level of significance for the Student t test comparing storage conditions at day 56: ns =*
357 *not significant (p>0.05); * p<0.05; ** p<0.01; *** p<0.001.*
358

359 At the beginning of the storage, WE2 and AE2 samples showed slightly higher PV

360 values than RF samples (p<0.05). This could be a consequence of the presence of trace

361 metals in the extracts (iron and copper) [18; 22] which promoted, together with the

362   temperature of processing (70-75 °C), oxidative reactions at an initial stage. During

363   storage, significant increments in PV were found in all samples (p<0.05).

364   At A0 storage conditions an increase in the oxidation rate, between day 1 and 56, was

365   found in WE2 (151%) and AE2 (154%) compared to RF (108%). It is well known that

366   interactions between lipid hydroperoxides and transition metals can induce the

367   formation of secondary oxidation compounds. Consequently, metal chelating capacity is

368   claimed as one of the important mechanisms of antioxidant activity [2, 29]. Regarding

369   this, several studies showed that *F. vesiculosus* extracts, containing phlorotannins, had

370   good ferrous ion-chelating capacity [12, 17]. In this sense, the presence of

371   phlorotannins may form complexes with metals and inactivate their catalytic effects in

372   promoting lipid hydroperoxide decomposition. Due to this antioxidant effect, an

373   accumulation of peroxide compounds in the extract containing samples might take place

374   and consequently lead to a lower formation of secondary oxidation compounds, as will

375   be discussed below.

376   Regarding A+, after 56 days of storage, while samples containing AE showed the

377   highest (AE2) or not significant differences (AE1) on PV, both WE samples had lower

378   PV than RF one (p<0.05). This could be due to the higher content of carotenoids in WE,

379   as carotenoids are well known inhibitors of free radical chain reactions caused by

380   photooxidation process [29].

381   Moreover, in the case of high temperature conditions it should be pointed out that a

382   higher oxidation rate, between day 1 and 35, was found in RF (185%), compared to

383   WE2 (102%) and AE2 (110%). The high content of phlorotannins in the extracts, with

384   radical scavenger activity could inhibit lipid oxidation initiation by radicals.

385   *3.2.3. Volatile compounds*

386 Odour deterioration of lipid containing products is caused mainly by the presence of

387 volatile secondary oxidation compounds, which have an impact on odour at extremely

388 low concentrations. Compounds formed from decomposition of lipid hydroperoxides

389 during storage can either react with unsaturated lipids to form stable and innocuous

390 alcohols, or undergo fragmentations into aldehydes and ketones causing rancidity in

391 unsaturated matrices [31]. Major volatile compounds identified from the headspace of

392 the fifteen samples throughout the storage were: four alkanals (pentanal, hexanal,

393 heptanal and octanal), two alkenals (*trans*-2-heptenal and 2-octenal) and two alcohols

394 (1-octen-3-ol and 2-ethyl-1-hexanol). These compounds represent groups of secondary

395 oxidation compounds resulting mainly from the autooxidation of oleic, linoleic and α-

396 linolenic acid [32, 33, 34]. Hexanal and 2-octenal showed the highest initial

397 concentrations (248±99 and 222±52 ng/g emulsion, respectively). However, others such

398 as pentanal and heptanal showed greater differences among samples and also more

399 evident variations during storage compared to their initial concentrations. This was the

400 reason why they were selected to follow their evolution during the whole storage (Fig.

401 4).

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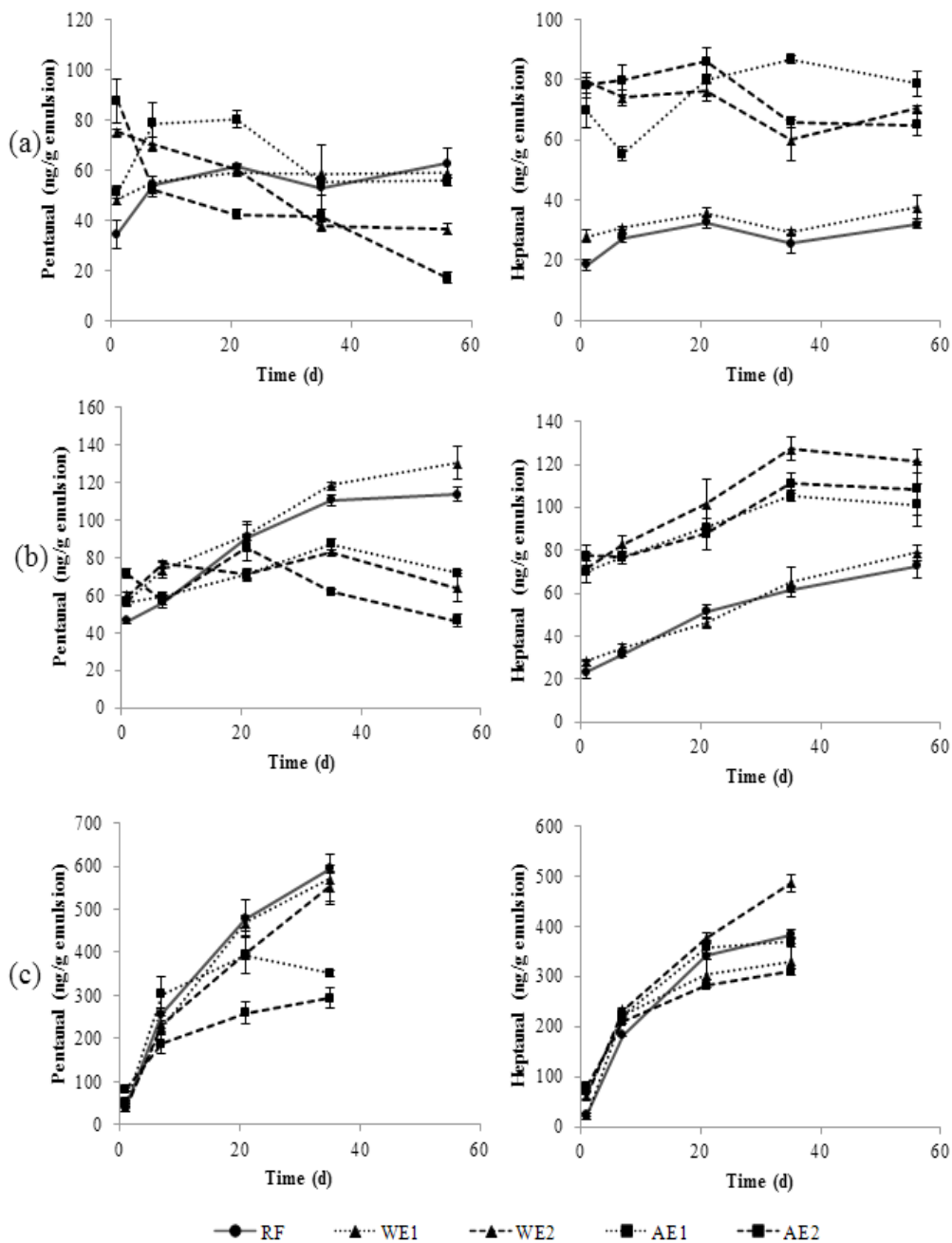
Fig. 4. Development of volatile compounds, pentanal and heptanal (ng/g emulsion), during the storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g). Error bars indicate SD of the measurements.

410   During accelerated storage conditions (A+ and H0), the peroxides decomposition

411   generated higher volatile amounts than in the A0 stored samples, so there was a higher

412   transformation rate from hydroperoxides to secondary oxidation products due to

413   thermo- and photooxidation processes. Furthermore, results showed that temperature

414   had significantly higher effect than light on the formation of volatile compounds, with

415   higher absolute amounts of both aldehydes at the end of the storage.

416   Regarding the presence of extracts, the concentration of pentanal and heptanal varied

417   between skin care emulsions at day 0 and during the storage. On one hand the highest

418   amounts of extracts contributed to increase the pentanal and heptanal concentration at

419   the beginning of all storages conditions. This could be due to the presence of these

420   compounds in the extract itself. Hermund et al. [18] observed higher amounts of some

421   volatile compounds (1-penten-3-ol and 1-penten-3-one) in milk emulsions containing *F.*

422   *vesiculosus* extracts.

423   On the other hand, in the samples with the highest extract content (WE2 and AE2),

424   pentanal showed significantly lower concentrations in all samples compared to RF at

425   the end of the storage (reduction up to 72% in AE2 samples at A0 storage conditions),

426   whereas heptanal amount was lower than RF only at the end of storage at high

427   temperature (19% reduction). On the other hand, the presence of antioxidant modified

428   the timing of volatile compound formation. Thus, even though the presence of extract at

429   the beginning of the storage resulted in higher amounts of pentanal and heptanal in all

430   samples, lower oxidation rates were observed during storage in these samples.

431   In particular, in the light stored samples, lower oxidation rates for pentanal and heptanal

432   were found in WE2 (6.7% and 69%, respectively) and AE2 (-35.2% and 40%,

433   respectively) compared to RF (144% and 211%, respectively). Moreover, at high

434   temperature, AE2 showed the best results against the formation of pentanal and

435   heptanal, with oxidation rates of 261% and 281%, respectively, compared to the rates

436   calculated for RF (1251% and 1419%). Finally, it is worth   noticing that at A0 stored

437   conditions, while RF showed an increment of pentanal (81%) and heptanal (71%)

438   between day 1 and 56, the highest extract concentration samples lead to a reduction

439   compared to their initial amounts. These results were in agreement with the

440   accumulative effect observed in PV in these samples. The presence of the extract

441   decreased the hydroperoxide decomposition rate to volatile secondary oxidation

442   compounds at all storage conditions, with AE2 being the most efficient extract. This

443   information helps to elucidate the antioxidant mechanism of those extracts, which may

444   influence the protection of the peroxides decomposition to secondary oxidation

445   products. However, more studies are needed to confirm these findings.

446

447   **4. CONCLUSIONS**

448   The type of antioxidant extract was a key factor in controlling oxidation processes of

449   skin care products influenced by light or temperature. Whereas both water and acetone

450   extracts of Icelandic *F. vesiculosus* showed (at 2 mg/g of emulsion) protective effect

451   against thermooxidation, only the water extract showed antioxidant activity against

452   photooxidation. Therefore, the presence of highly antioxdative phlorotannin (radical

453   scavenging activity and iron-chelating capacity) in the extracts presumably contributed

454   to decreasing the lipid oxidation. Moreover, the higher carotenoids content in the water

455   extract could inhibit free radical chain reactions caused by the photooxidation process.

456   The Icelandic *F. vesiculosus* extracts were effective in protecting highly-unsaturated

457   skin care emulsions but gave rise to colour changes particularly when stored at high

458   temperature.

459

5. ACKNOWLEDGEMENTS

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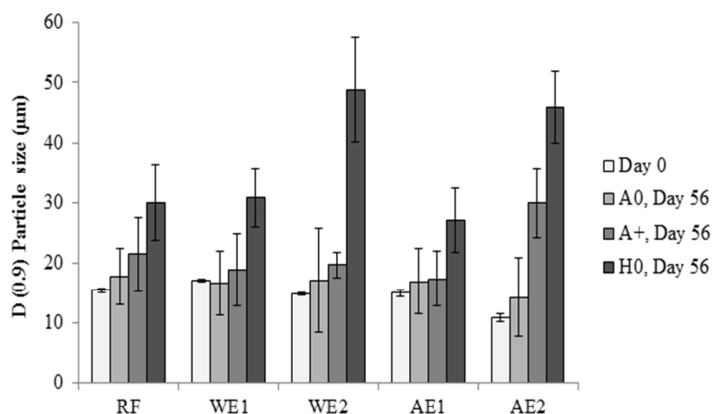
Fig. 1. Droplet size distribution on cosmetic emulsions with or without F. vesiculosus extract (water or acetonic) after 56 days of storage under accelerated conditions or at room temperature and dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); A0, darkness and room temperature; A+, light and room temperature; H0, darkness and high temperature).
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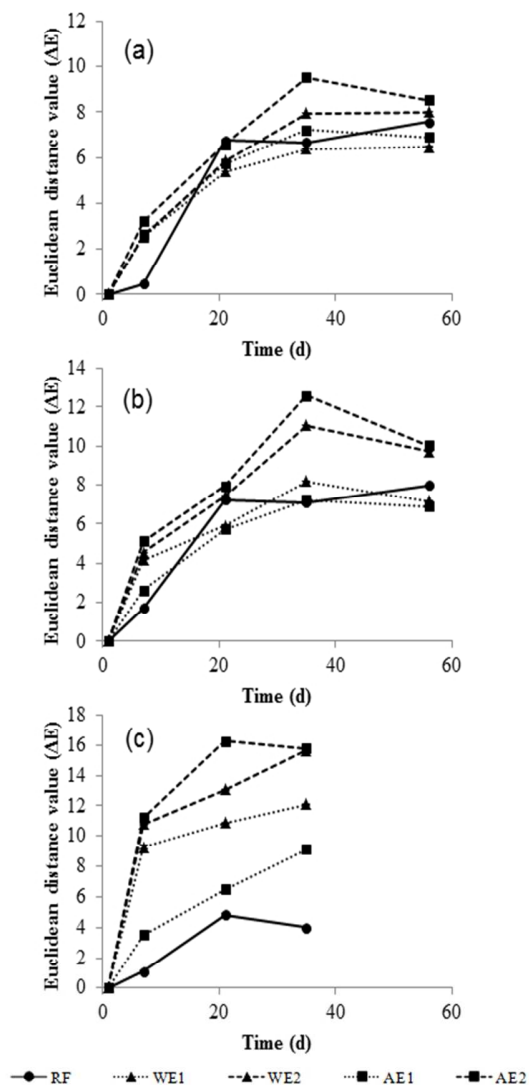
Fig. 2. Euclidean distance value of the cosmetic emulsions calculated along the storage. It compares color at each time of storage to its color at day 0. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g)). Error bars indicate SD of the measurements.
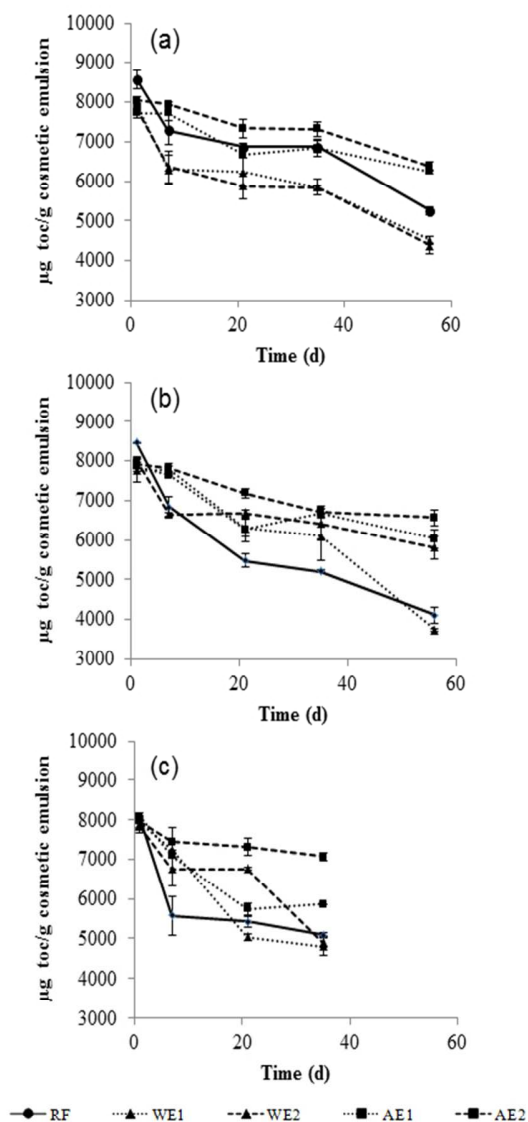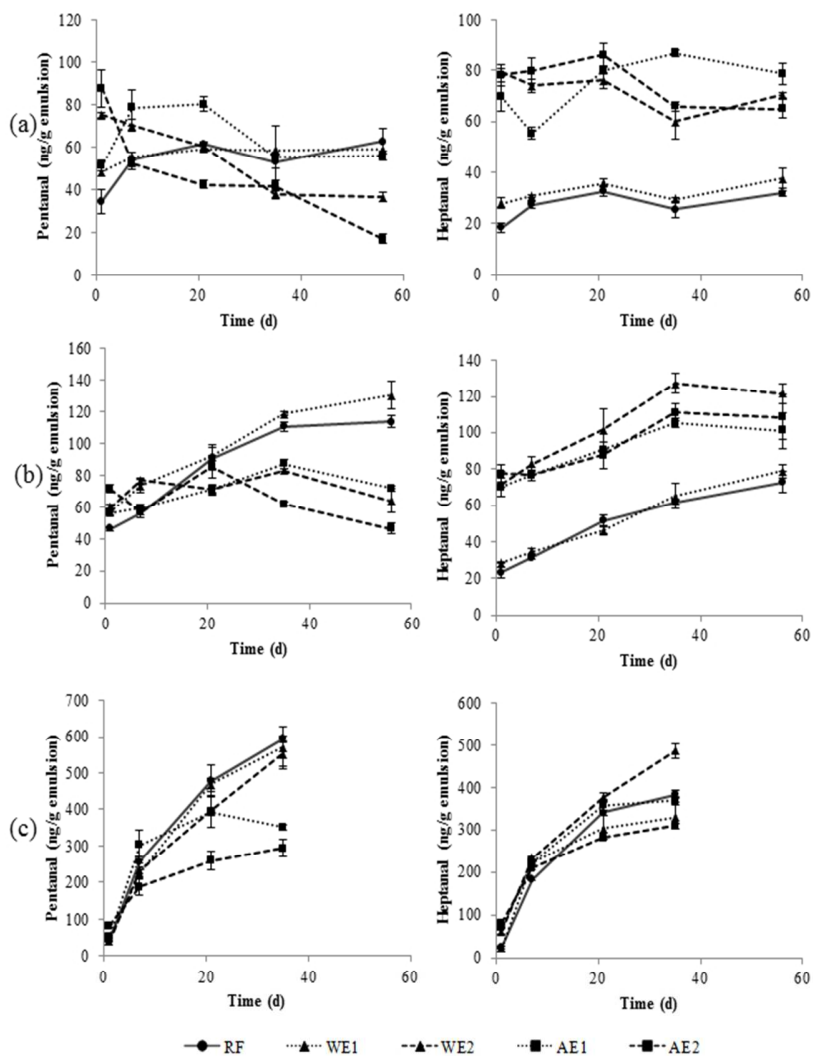190x254mm (96 x 96 DPI)

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Fig. 3. Total tocopherol content (µg tocopherol/g cosmetic emulsion) in emulsions with WE or AE including a control without any extract during storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g)). Error bars indicate SD of the measurements.
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Fig. 4. Development of volatile compounds, pentanal and heptanal (ng/g emulsion), during the storage. (a) A0, room temperature and darkness; (b) A+, room temperature and light; (c) H0, high temperature and darkness. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g)). Error bars indicate SD of the measurements.
190x254mm (96 x 96 DPI)

Table 1. Formulation of the different samples.

Water phase	
Demineralized water	52.5 g/ 100 g
Aloe vera water	10.0 g/ 100 g
Glycerin	6.3 g/ 100 g
MF fat	3.6 g/ 100 g
Natriumbenzoat	0.6 g/ 100 g
F. vesiculosus extract (water or acetonic)	1 or 2 mg/g

Oily phase	
Almond oil	21.8 g/ 100 g
Lanette wax	2.0 g/ 100 g
VE fat	1.8 g/ 100 g
Vitamin E	0.9 g/ 100 g

Table 2. Effect of adding water or acetonic *F. vesiculosus* extract on PV (meq O$_2$/kg oil) of cosmetic emulsions under accelerated stored conditions or room temperature and dark. (RF, reference; WE1, water extract (1 mg/g); WE2, water extract (2 mg/g); AE1, acetone extract (1 mg/g); AE2, acetone extract (2 mg/g); S.C, storage condition; A0, darkness and room temperature; A+, light and room temperature; H0, darkness and 40 °C.)

<div align="center">Peroxide value (meq O$_2$/kg oil)</div>

	Day 0	S.C	Day 7	Day 21	Day 35	Day 56
RF	5.52 ± 0.51^{A}	A0	6.85 ± 0.29^{Ba}	7.31 ± 1.00^{Aa}	9.48 ± 0.45^{Aa}	11.62 ± 0.31^{A}
		A+	8.22 ± 0.52^{Ab}	11.71 ± 1.07^{Bb}	13.11 ± 1.19^{ABb}	18.93 ± 0.63^{B}***
		H0	11.49 ± 0.84^{ABc}	11.65 ± 0.29^{Ab}	15.01 ± 0.78^{ABb}	
WE1	5.19 ± 0.29^{A}	A0	6.09 ± 0.04^{ABa}	6.97 ± 0.45^{Aa}	8.53 ± 0.36^{Aa}	10.77 ± 1.25^{A}
		A+	8.11 ± 0.05^{Ab}	10.58 ± 0.59^{ABb}	12.37 ± 0.70^{ABb}	15.67 ± 1.29^{A}**
		H0	10.11 ± 1.11^{Ac}	13.08 ± 1.33^{Ac}	14.31 ± 0.07^{Ac}	
WE2	6.58 ± 0.69^{B}	A0	12.89 ± 0.67^{Da}	11.95 ± 0.22^{Ba}	13.96 ± 0.32^{Ca}	17.67 ± 1.38^{C}
		A+	14.08 ± 0.21^{Da}	12.40 ± 0.69^{Ba}	14.38 ± 1.28^{Ba}	$16.42\pm1.42^{A\ ns}$
		H0	13.05 ± 1.60^{BCa}	11.38 ± 0.19^{Aa}	13.97 ± 0.19^{Aa}	
AE1	6.28 ± 0.65^{B}	A0	14.26 ± 0.29^{Ea}	13.36 ± 1.03^{Bb}	15.34 ± 0.35^{Db}	18.78 ± 0.71^{C}
		A+	13.16 ± 0.32^{Ca}	10.90 ± 1.15^{ABa}	10.34 ± 0.52^{Aa}	23.97 ± 0.49^{C}***
		H0	13.73 ± 1.71^{Ca}	16.58 ± 1.61^{Bc}	16.47 ± 1.73^{Bb}	
AE2	6.63 ± 0.75^{B}	A0	11.31 ± 0.28^{Cb}	17.03 ± 0.47^{Cb}	12.36 ± 0.87^{Ba}	15.14 ± 0.95^{B}
		A+	9.58 ± 0.23^{Ba}	9.43 ± 0.35^{Aa}	17.36 ± 1.99^{Cb}	18.89 ± 0.55^{B}**
		H0	10.94 ± 0.21^{ABb}	10.43 ± 0.78^{Aa}	14.05 ± 1.05^{ABab}	

Different capital letters in the same column denote significant differences between samples for each storage condition (p<0.05)

Different small letters in the same column denote significant differences among storage conditions for each sample (p<0.05)

Level of significance for the Student t test comparing storage conditions at day 56: ns = not significant (p>0.05); * p<0.05; ** p<0.01; *** p<0.001.
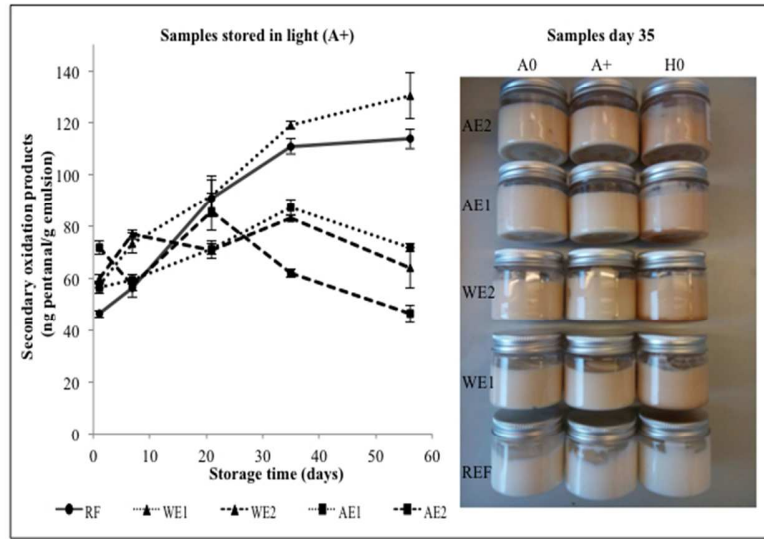
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Graphical abstract. Skin care emulsions were exposed to light (A+) or elevated storage temperatures (H0) to promote lipid oxidation during storage. Control samples were stored at ambient temperatures in the dark (A0). Two Icelandic brown alga *Fucus vesiculosus* extracts, water (WE) and acetone (AE) extract, where added as natural antioxidants to control lipid oxidation and ensure a stable product. Both extract (depending on concentration, 1 or 2) decreased photooxidation of skin care emulsions compared to the reference (REF) without added extract, as decreased formation of secondary volatile oxidation products was observed. However, the presence of extracts in the skin care emulsions induced remarkable colour changes during storage. Moreover, samples exposed to elevated storage temperatures increased oil droplet size and resulted in disrupted emulsion structure (syneresis) by day 35.

PAPER VI

Structure dependent antioxidant capacity of phlorotannins from Icelandic *Fucus vesiculosus* by UHPLC-DAD-ECD-QTOFMS

Hermund, DB, Plaza, M, Turner, C, Jónsdóttir, R, Kristinsson, HG, Jacobsen, C, & Nielsen, KF

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Abstract: Brown algae are characterized by the presence of a specific group of polyphenolic compounds, the phlorotannins, which have been found to possess high in vitro antioxidant capacity especially DPPH radical scavenging activity due to the high number of hydroxyl groups. Whereas, the overall antioxidant capacity of crude brown algae extracts have been widely studied and extracts have been fractionated in an attempt to study the importance of the phlorotannin structure on their antioxidant performance. The antioxidant capacity of individual phlorotannins has not yet been explored.
The aim of this study was therefore to determine the structure dependant antioxidant capacity of phlorotannins from Icelandic brown algae, Fucus vesiculosus. Hence, the antioxidant capacity of individual phlorotannins was determined by an on-line method using liquid chromatography and an electrochemical detector followed by quadrupole Time of Flight mass spectrometry (UHPLC-DAD-ECD-QTOFMS).
Tentative structural elucidation of 13 phlorotannin isomers from EAF was obtained by LC-DAD-QTOFMS, ranging from 374 to 870 Da. On-line determination of antioxidant capacity of the individual phlorotannins generally showed that low molecular phlorotannins exhibited higher antioxidant capacity and also that the capacity decreased with polymerisation.

Cover Letter

Food Research International
21 March 2016

Manuscript for special issue in Food Research International

Dear editor

I hereby submit a short communication for the special issue on "Microalgae and Seaweeds as Potential Source of Valuable Nutrients, Food Additives and Nutraceuticals for Human and Animal Consumption".

The goal of this research is to identify and characterize highly antioxidative phlorotannins derived from Icelandic brown alga *Fucus vesiculosus.* This was obtained by advanced analytical methods including on-line determination of the antioxidant capacity of individual phlorotannin isomers using UHPLC-DAD-ECD-QTOFMS. Tentative structural elucidation and antioxidant capacity of 9 phlorotannin isomers were obtained, which provide knowledge on the structure/antioxidant relationship of these compounds. The results have high novelty and are a valuble contribution to the research field.

The main manuscript is within your guidelines for short communication papers (approximately 3000 words) and includes 1 table and 2 figures, which are in separate files. Included is supplementary material (Fig. S1) on MS/MS fragmentation of one of the identified phlorotannin isomers.

I sincerely hope that you will consider this manuscript for publication in the special issue.

Best regards,
Ditte B. Hermund
Main author

Highlights

- Tentative structural elucidation of 13 phlorotannin isomers from *Fucus vesiculosus*
- On-line determination of antioxidant capacity of 9 individual phlorotannins
- Generally the antioxidant capacity decreased with polymerization
- Structure dependant antioxidant capacity was found

1   Structure dependent antioxidant capacity of phlorotannins from Icelandic *Fucus vesiculosus*

2   by UHPLC-DAD-ECD-QTOFMS

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4   Ditte B. Hermund[1], Merichel Plaza[2], Charlotta Turner[2], Rosa Jónsdóttir[3], Hordur G. Kristinsson[3],

5   Charlotte Jacobsen[1], Kristian Fog Nielsen[4*]

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16   Key words

17   Brown algae, screening, free radical scavenging, MS/MS

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19 Abstract

20 Brown algae are characterized by the presence of a specific group of polyphenolic compounds, the

21 *phlorotannins*, which have been found to possess high *in vitro* antioxidant capacity especially

22 DPPH radical scavenging activity due to the high number of hydroxyl groups. Whereas, the overall

23 antioxidant capacity of crude brown algae extracts have been widely studied and extracts have been

24 fractionated in an attempt to study the importance of the phlorotannin structure on their antioxidant

25 performance. The antioxidant capacity of individual phlorotannins has not yet been explored.

26 The aim of this study was therefore to determine the structure dependant antioxidant capacity of

27 phlorotannins from Icelandic brown algae, *Fucus vesiculosus*. Hence, the antioxidant capacity of

28 individual phlorotannins was determined by an on-line method using liquid chromatography and an

29 electrochemical detector followed by quadrupole Time of Flight mass spectrometry (UHPLC-DAD-

30 ECD-QTOFMS).

31 Tentative structural elucidation of 13 phlorotannin isomers from EAF was obtained by LC-DAD-

32 QTOFMS, ranging from 374 to 870 Da. On-line determination of antioxidant capacity of the

33 individual phlorotannins generally showed that low molecular phlorotannins exhibited higher

34 antioxidant capacity and also that the capacity decreased with polymerisation.

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36

1. Introduction

New research confirms the antioxidant potential of Icelandic brown algae *Fucus vesiculosus* extracts as natural antioxidants in fish muscle and foods enriched with fish oil, to limit oxidation of marine polyunsaturated fatty acids, like EPA and DHA (eicosapentaenoic and docosahexaenoic acid), in these types of products (Halldorsdottir et al., 2014; Hermund et al., 2015; Honold et al., 2015).

Brown algae are characterized by the presence the polyphenolic phlorotannins, which have been found to possess high *in vitro* antioxidant activity (Koivikko et al., 2005; Wang et al, 2009). These compounds are a subgroup of tannins, which are formed by polymerization of phloroglucinol units (PGU) (1,3,5-trihydroxybenzene, M_w 126 Da) (Ragan and Glombitza, 1986). *Fucus vesiculosus* most likely contain low molecular weight phlorotannins (<1200 Da) (Steevensz et al., 2012). According to Martínez and Castañeda (2013) there are three main groups of phlorotannins: 1) fucols, 2) phloroethols and 3) fucophloroethols. Fucols are phlorotannin polymers in which the PGUs are connected only by C-C (phenyl linkage) bonds in meta position. Phloroethols consist of PGUs, which are linked only by C-O-C (arylether) bonds. The linear phloroethols may have ortho-, meta- or para-oriented biphenyl ether bridges. Fucophloroethols are a mixture of both biaryl and aryl-ether bonds allowing a variety of compounds in linear, branched and heterocyclic fashions. Due to the high complexity of phlorotannin structures only few studies have dealt with their detection. Typically, the phlorotannins are determined as a total content by Folin Ciocalteu reagents by measuring the reductants present in the extracts as an estimate for the total phlorotannin content (TPC) (Singleton and Rossi, 1965). However, more in-depth studies of identification and characterization of phlorotannins are needed.

The availability of advanced chromatographic and mass spectrometric techniques gives the possibility for tentative identification of phlorotannins. Wang et al. (2012) and Heffernan et al.

61 (2015) both characterized phlorotannins extracted from *F. vesiculosus* using such techniques.

62 Heffernan et al. (2015) used Liquid Chromatography with tandem MS for profiling fractions of

63 phlorotannins from brown algae. Wang et al. (2012) performed simple purification of 80% (v/v)

64 ethanol extract made from *F. vesiculosus* by liquid-liquid partitioning, e.g. using ethyl acetate, to

65 obtain fractions rich in phlorotannins. Hereafter, these fractions were submitted to semi-preparative

66 column chromatography before high performance liquid chromatography (HPLC) electrospray

67 (ESI) coupled to MS and MS/MS analyses and tentative identification of phlorotannins were

68 obtained. Both these studies used antioxidant assays to study antioxidant properties such as radical

69 scavenging activity of the extract/fractions in order to relate the structure of the phlorotannins to the

70 antioxidant capacity. However, by applying an on-line method, which can provide tentative

71 identification of phlorotannins and at the same time determine the antioxidant capacity of each

72 phlorotannin it would be possible to provide an in-depth knowledge of which phlorotannins

73 contribute to the overall antioxidant capacity of these extracts. Plaza et al. (2013) showed that by

74 coupling an electrochemical detector (ECD) to an HPLC it was possible to determine the

75 antioxidant capacity of phenolic compounds from apples on-line.

76 The aim of this study was evaluate the structure dependant antioxidant capacity of phlorotannins by

77 a fast screening method. This was performed on a purified phlorotannin-rich fraction (EAF) from

78 Icelandic brown algae *F. vesiculosus*, which in previous studies have shown great potential as

79 natural antioxidant in food emulsions (Hermund et al, 2015). The individual phlorotannins and their

80 antioxidant capacity were identified and characterized by HPLC-DAD-ECD-QTOFMS analysis of

81 EAF.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals were of analytical grade. Formic acid and acetic acid were from Merck (Darmstadt, Germany). Phloroglucinol standard was purchased from Sigma-Aldrich (St. Louis, MI, USA). The ultrapure water used was obtained from a Milli-Q (Millipore, Billerica, MA, USA) instrument.

2.2. Algae material, solvent extraction, partitioning and fractionation

Solvent extraction and partitioning was performed by Matís in Iceland according to Wang et al. (2012). The seaweed was collected from intertidal water in the Hvassahraun coastal area near Hafnarfjordur, southwestern Iceland, in September 2011. At the collecting spot the seaweed was washed with clean seawater to remove salt, epiphytes and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water and wiped with paper towel. The samples were freeze-dried, pulverised into powder and stored at -80°C prior to extraction.

An 80% (v/v) aqueous ethanol extract (EE) was subjected to liquid-liquid partitioning to produce an ethyl acetate fraction (EAF). To obtain EE, five grams of freeze-dried algal powder were dispersed in 100 mL 80% (v/v) ethanol and incubated in the platform shaker (InnovaTM 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2168g for 10 min at 4°C and filtered with Whatman no. 4 filter paper to abtain a liquid extract. EAF was produced by collecting 100 mL EE and concentrating it to a small volume by evaporation of the ethanol. The concentrate was suspended in a mixture of methanol and water (40:30, v/v) and partitioned three times with n-hexane, ethyl acetate, and 1-butanol successively. Hereafter, the solvents were removed and the remaining fractions were freeze-dried. Thereby, four fractions were obtained, the n-hexane, ethyl acetate (EAF), and 1-butanol soluble fractions and an

5

106 aqueous residue. Only the EAF was used in this study because of its high TPC (Wang et al., 2012).

107 EAF was stored as freeze-dried powder at -80°C until used for analysis. Prior to analysis the

108 powders was dissolved in DM water.

109

110 ## 2.3. HPLC-DAD-ECD analysis

111 *Instrumentation.* The method setup was based on a similar method used for polyphenols and

112 phenolic acids (Plaza et al., 2013; Safafar et al., 2015). It consisted of an UltiMate-3000$^{®}$ HPLC

113 system (Dionex, Thermo Fisher, Germering, Germany) with a photodiode array detector (DAD).

114 The detection wavelengths used were: 200, 280, 350, 370, and 520 nm. An ECD instrument

115 (Bioanalytical System Inc., West Lafayette, IN, USA) was attached just after the DAD to do online

116 amperometric detection. The setup has been described in Plaza et al. (2013).

117 *Chromatographic separation.* Separation was obtained on a Phenomenex Prodigy 3 μm ODS 3

118 150x2mm column. The mobile phase consisted of ammonium formate buffer (A) (pH 3, 20 mM

119 formic acid), 60 mM ($NH_4HCOO/HCOOH$) in water; and acetonitrile (B) (20 mM formic acid).

120 The mobile phases were purged with nitrogen to remove oxygen. The gradient elution analysis

121 program was as follows: 0-2 min, 0% (B); 2-16 min, increasing to 40% (B); 16-18 min, increasing

122 to 100% (B), with 17 min of post-time at a flow rate of 0.3 mL/min. All compounds had eluted

123 within the first 17 min and therefore the chromatograms are of this duration. The column

124 temperature was set at 25°C, the injection volume was 2 μL, and the vial tray was held at 4°C. For

125 instrument validation, phloroglucinol standard (1 mg/mL for HPLC) and the retention time was

126 used for control. All analyses were conducted in triplicates.

127

2.4. UHPLC-DAD-QTOFMS analysis

Instrumentation. Ultra-high performance liquid chromatography-DAD-quadruple time of flight mass spectrometry (UHPLC-DAD-QTOFMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a DAD coupled to an Agilent 6545 QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source (Kildgaard et al., 2014). MS and MS/MS were performed at m/z 100-1600 and auto-MS/MS was done at 10, 20, and 40 eV. Hexakis (2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK) at 921.23 was used as lock mass in positive and negative mode as the $[M+H]^+$ and $[M+HCOO]^-$ ions respectively.

Chromatographic separation. Separation was obtained similar to the method used for HPLC-DAD-ECD analysis with some alterations. The gradient elution analysis program was as follows: 0-2 min, 0% (B); 2-16 min, increasing to 40% (B); 16-18 min, increasing to 100% (B), with 17 min of post-time at a flow rate of 0.3 mL/min. All compounds had eluted within the first 17 min and therefore the chromatograms are of this duration. The column temperature was set at 25°C, the injection volume was 2 μL. For instrument validation, phloroglucinol standard (PG) (0.1 mg/mL for LC) and the retention time was used for control.

2.5. Data analysis

The areas (nAs) of the ECD responses for the EAF were calculated (mean±SD). UHPLC-DAD-QTOF data analysis was performed in MassHunter 6.00 where the base peak chromatograms (BPC) were made with major background ions subtracted. For finding known phlorotannins the Find-By-Formula function in Masshunter was used searching for the following singly charged adducts: ESI^+, $[M+H]^+$ and $[M+Na]^+$; ESI^-, $[M-H]^-$, $[M+HCOO]^-$.

3. Results and discussion

3.1. Structural elucidation of phlorotannins

The Extracted Ion Chromatograms (EIC) of deprotonated molecular ions ([M-H]⁻) from the most common phlorotannins found in literature (eckol (*m/z* 371.0409), fucophloroethol (*m/z* 373.0565), 7-phloroeckol (*m/z* 495.0569), fucodiphloroethol (*m/z* 497.0725), phlorofucofuroeckol (*m/z* 601.0624), fucotriphloroethol (*m/z* 621.0886), dieckol (*m/z* 741.0733), and fucophloroethols with six (*m/z* 745.1046), and seven PGUs (*m/z* 869.1207)), were used for the study of phlorotannins in EAF by UHPLC-DAD-QTOFMS. Furthermore the elemental compositions were verified by the accurate mass (± 5 ppm) and isotopic patterns.

In Fig. 1. the Base Peak Chromatogram (BPC) of EAF is shown together with the UV chromatogram and EICs of the selected ions. In the EICs, some peaks were overlapping due to insource fragmentation (i.f.) giving false/positive results when consulting MS-data, e.g. one peak in EIC of *m/z* 373 was found to be an insource fragmentation of *m/z* 497. When taking this into account the EICs revealed well-defined and abundant ions of 11 (1-11) compounds tentatively corresponding to phlorotannins and corresponding with the UV chromatogram, and two compounds (12-13), which were only found in trace amounts and were not so well-defined, neither by UV. There were no responses in the UV chromatogram, which did not correspond to the studied ions.

The MS study of the ions allowed the detection of several isomers. The isomers were studied in negative ionization mode to investigate the fragmentation patterns with the aim of getting closer to an exact structural identification of the isomers.

Compounds 6, 7 and 8, in negative mode, showed similar fragmentation patterns in which some ions are characteristic of phlorotannins fragmentation, e.g. for compounds with losses of one and two water molecules (-18.0101 (m/z 603.0778) and -36.0209 (m/z 585.0670), respectively), loss of 1 PGU and water (-126.0324, -18.0101 (m/z 477.0454)), and loss of 2 PGUs and water, as well as

8

the presence of deprotonated molecular ion of phloroglucinol (m/z 125.0133). Thus, these three

compounds are suggested to be phlorotannins composed of five PGUs, possibly isomers of

fucotriphloroethol. In the supplementary material (Fig. S1), the structure of fucotriphloroethol

(linear) and suggested fragmentation of this phlorotannin are shown. It is most likely that the loss of

one and two water occurs first, followed by fragmentation from the ether-end due to the higher

lability of this bond compared to the phenyl-linkage.

In Table 1 the fragmentation patterns in negative mode of the 13 identified phlorotannin compounds

(including isomers) are listed. Isomers of phlorotannins trimers with [M-H]$^-$ at *m/z* 373 (compound

1-3) were observed, which correspond tentatively to fucophloroethol. Isomers of phlorotannins

tetramers with [M-H]$^-$ at *m/z* 497 (compound 4-5) were observed, which correspond tentatively to

fucodiphloroethol. Furthermore, isomers with [M-H]$^-$ at *m/z* 745 (compound 9-11) and *m/z* 869

were tentatively identified as fucophloroethols with six or seven PGUs, respectively. Hydrogen

migration was observed (noted as either +2 or -2 in Table 1) in some of the fragments. Even though

the fragmentation patterns of the isomers showed some differences, indicating structural diversity, it

was not possible to make further elucidation of the structures. Further structural identification of the

isomers would require severe purification of the extracts as well as NMR (nuclear magnetic

resonance). Heffernan et al. (2015) also using MS and no NMR, found that *F. vesiculosus* contained

phlorotannins in the range of 3 to 16 PGU, with the most abundant phlorotannins at a low molecular

weight range, e.g. *m/z* 497 (4PGUs), *m/z* 745 (6PGU) and *m/z* 869 (7PGUs).

3.2. Structure dependent antioxidant capacity of phlorotannins

Along with the identification of phlorotannins in EAF, on-line detection of the antioxidant capacity

of individual phlorotannins was carried out by HPLC-DAD-ECD. It was possible to detect

compound 2 to 11 by UV (Fig. 2). However, for some compounds it was not possible to distinguish

the ECD response, e.g. compound 3 and 9 have different composition, but could not be separated in

the ECD, hence the ECD response of these two compounds were not determined. Non-separable

ECD responses of compounds with the same composition were though determined. Hence,

determination of antioxidant capacity of individual phlorotannins was only carried out for

compound 2 to 11, excluding compound 3 and 9, by calculating the ECD response (nAs). The

results are shown in Table 1.

Shibata et al. (2008) and Audibert et al. (2010) found that the radical scavenging activity of partially

semi-purified extracts of phlorotannins obtained from brown algae was related to the content of

phlorotannins and to their molecular weight and that the increase of molecular weight of the

isolated phlorotannins led to a decrease in the antioxidant capacity. Both found that the increase in

molecular weight of the isolated phlorotannins led to a decrease in antioxidant capacity (DPPH

radical scavenging).

The present study is the first of its kind to evaluate antioxidant capacity of individual phlorotannins

and not just fractions with phlorotannins in a specific molecular weight range. Compound 2, an

isomer of fucophloroethol (3 PGUs) showed the highest antioxidant capacity, and the capacity

seemed to decrease with increased polymerization of phlorotannins (Table 1). However, there was

one exception as Compound 6 consisting of 5 PGUs showed higher antioxidant capacity than

phlorotannins consisting of 4 PGUs (Compound 4 and 5).

These results indicate that it is the availability of hydroxyl groups more than the polymerization

which determines the antioxidant capacity of the phlorotannins. It can be hypothesised that large

phlorotannin polymers might fold in a way, which encloses the OH-groups inside the structure, and

therefore poorer antioxidant capacity of large phlorotannins was observed. However, the enclosed

structure and unavailable OH-groups are dependent on the branching of the phlorotannins, therefore

one isomer of phlorotannin consisting of 5 PGUs showed higher antioxidant capacity compared

224 with other isomers, which might be branched in a different way that favours folding of the

225 compound in a way which decreases their antioxidant capacity. As mentioned, this has to be

226 verified with additional NMR analysis.

227

4. Concluding Remarks

229 Tentative structural elucidation of 13 phlorotannin isomers from EAF was obtained by UHPLC-

230 DAD-QTOFMS ranging from 374 to 870 Da. It was not possible to determine the structural

231 differences between isomers, though the fragmentation patterns obtained showed clear differences

232 presumably due to different branching of the phlorotannins. On-line determination of antioxidant

233 capacity of the individual phlorotannins generally showed that low molecular weight phlorotannins

234 exhibited higher antioxidant capacity and also that the capacity decreased with polymerisation. This

235 method could be used as a fast screening of complex seaweed extracts to identify the presence of

236 highly antioxidative phlorotannins, e.g. isomers of fucophloroethol (3 PGUs).

237

5. Acknowledgments

243

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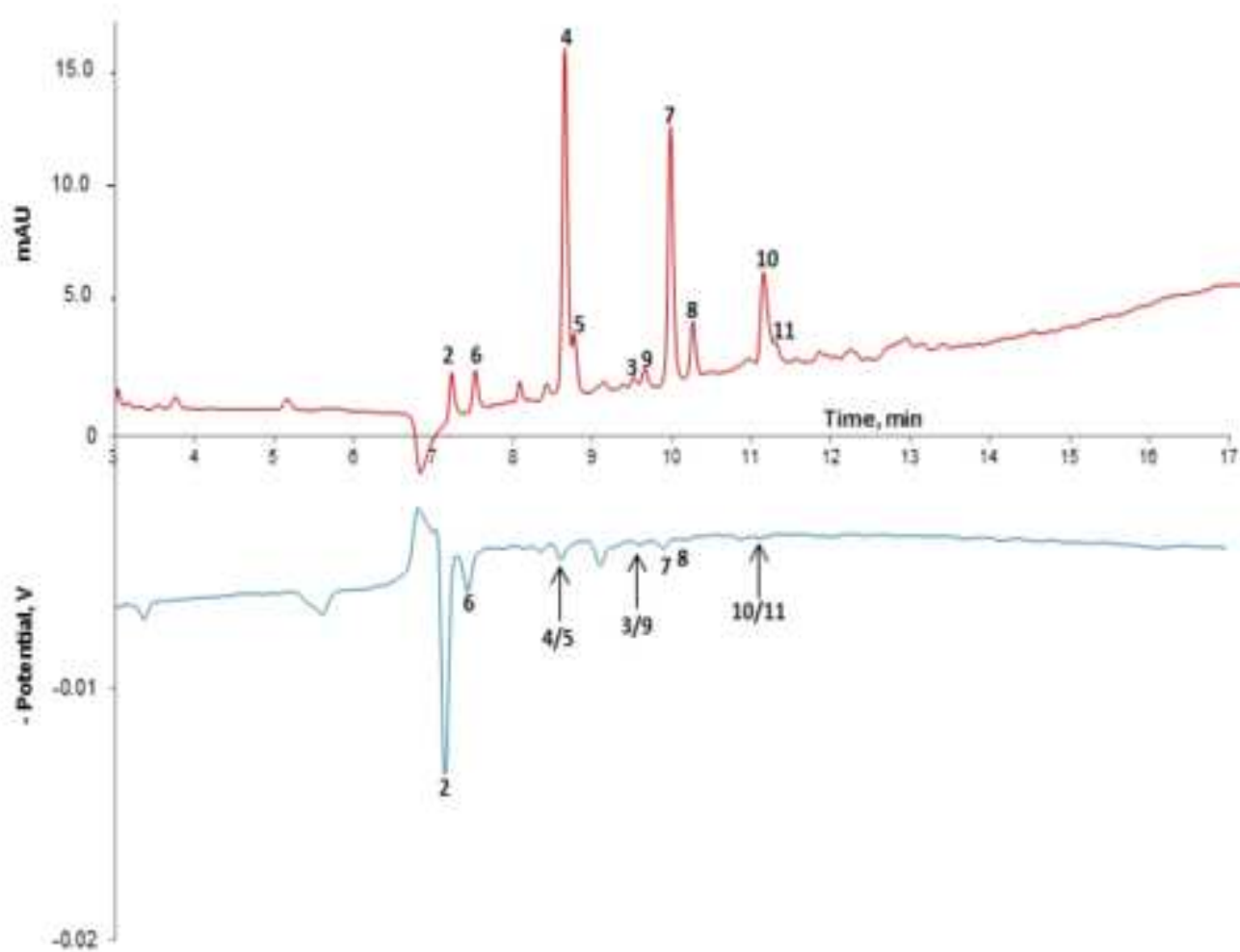
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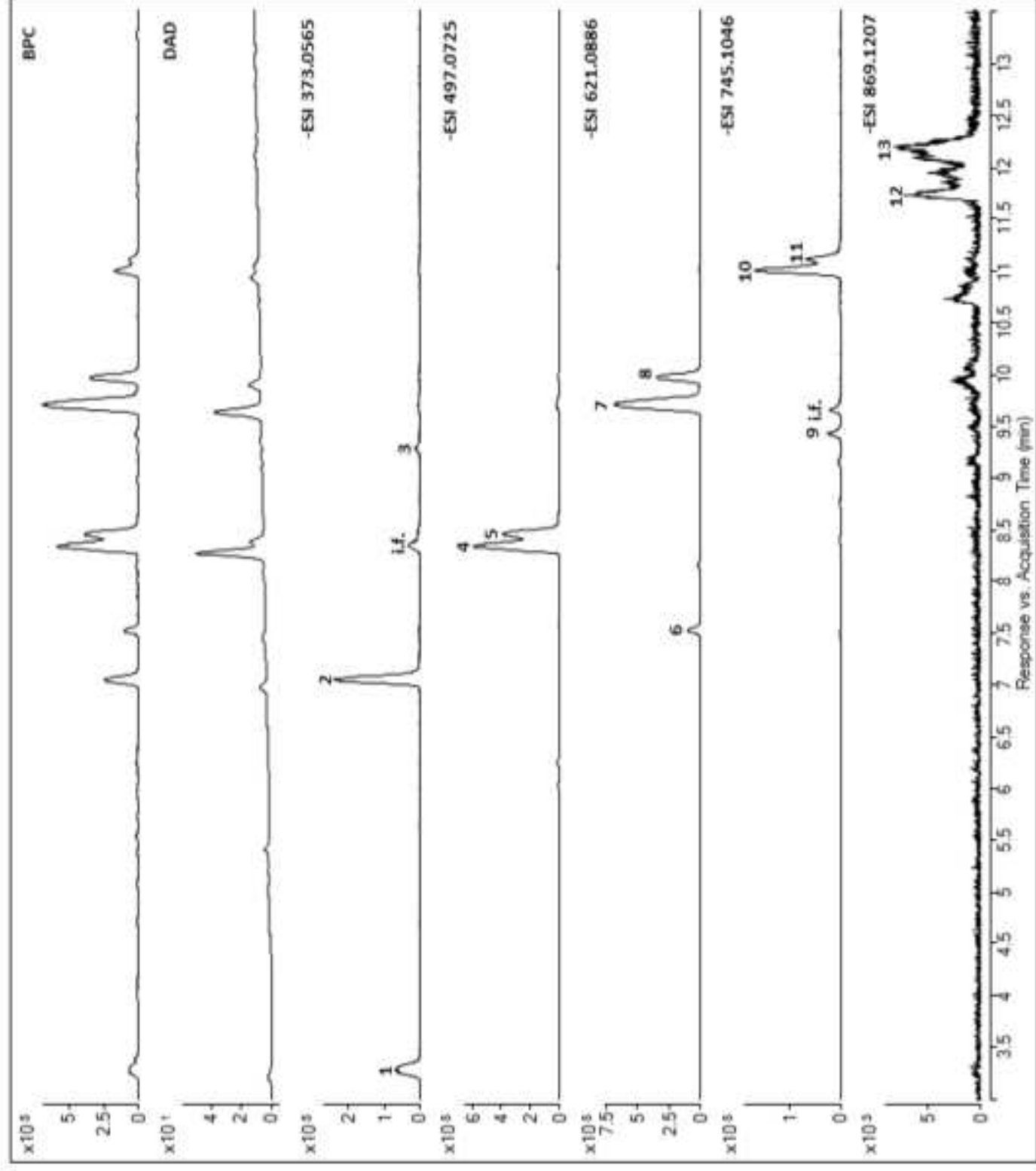
Fig. 1. Base Peak chromatogram (BPC), UV chromatogram (DAD) and Extracted Ion Chromatograms (-EIC) of phlorotannins from EAF. [M-H]$^-$ monoisotopic m/z: 1-3,373.0565; 4-5, 497.0725; 6-8, 621.0886; 9-11, 745.1046; 12-13, 869.1207 ($\pm$ 5 ppm). i.f.=insource fragmentation

Fig. 2. UV chromatogram and amperogram corresponding to the HPLC-DAD-ECD analysis of EAF. The UV chromatogram at 280 nm (red) and amperogram (blue) in negative potential, -V. Compound 2 to 11 are numbered

Table 1. Elementary composition, retention time, UV (nm), [M-H]⁻, MS² [M-H]⁻ data and ECD responses (nAs) for compound (C) 1 to 13 from EAF

C	Elementary Composition	RT (min)	UV (nm)	[M-H]⁻	ppm	MS/MS fragmentation pattern	ECD [nAs]
1		3.256	205, 274sh	373.0564	-0.27	355.0448 (-H_2O), 329.0184, 311.0554, 305.0575, 287.0556, 269.0460, 243.0689, 229.0135 (-1PGU, -H_2O), 214.2045, 207.0282, 181.0493, 165.0192, 139.0370, 125.0244	-
2	$C_{18}H_{14}O_9$	7.048	210, 272sh	373.0560	-1.34	355.0459 (-H_2O), 329.0659, 305.0661, 287.0558, 261.0764, 243.0667, 231.0286 (-1PGU, -H_2O, +2), 216.0062, 205.0506, 189.0558, 165.0191, 149.0239, 141.0187, 124.0157 (-1)	63.10±1.54
3		9.324	213	373.0590	6.70	329.0381, 311.0579, 259.0246, 247.0243 (-1PGU), 229.0140 (-1PGU, -H_2O), 219.0287, 201.0190, 177.0211, 161.0223, 141.0191, 125.0248	-
4		8.335	208, 273sh	497.0729	0.80	479.0619 (-18), 453.0814, 435.0720, 413.0511, 395.0395, 371.0411 (-1PGU), 353.0307 (-1PGU, -18), 335.0200 (-1PGU, -2H_2O), 325.0353, 309.0404, 287.0191, 267.0305, 247.0242 (-2PGU, +2), 229.0145 (-2PGU, -H_2O, +2), 219.0299, 203.0348, 191.0346, 165.0190, 139.0032	
5	$C_{24}H_{18}O_{12}$	8.471	210, 271sh	497.0717	-1.61	479.0612 (-H_2O), 453.0792, 435.0693, 411.0718, 395.0363, 371.0404 (-1PGU), 353.0292 (-PGU, -H_2O), 339.0500, 327.0507, 309.0390, 283.0250, 267.0305, 247.0242 (-2PGU, +2), 229.0133 (-2PGU, -H_2O, +2), 205.0496, 165.0199, 139.0031, 125.0234	5.63±0.59
6		7.535	211, 275sh	621.0880	-0.97	603.0769 (-H_2O), 577.0974, 541.0765, 477.0423 (-1PGU, -H_2O), 455.0613, 433.0548, 413.0507, 373.0526 (-2PGU, +2), 343.0442, 311.0205, 287.0200, 247.0262, 207.0291 (-3PGUs, -2H_2O), 165.0189, 125.0241	24.63±1.54
7	$C_{30}H_{22}O_{15}$	9.708	208, 275sh	621.0891	0.81	603.0782 (-H_2O), 585.0654 (-2H_2O), 559.0864, 537.0660, 479.0607 (-1PGU, -H_2O, +2), 433.0539, 371.0400 (-2PGUs, +2), 353.0301 (-2PGUs, -H_2O, +2), 335.0194 (-2PGUs, -2H_2O, +2), 309.0401, 283.0233, 249.0403, 229.0140, 205.0503 (-3PGUs, -2H_2O, -2), 163.0404, 139.0034	2.52±0.16
8		9.964	212, 273sh	621.0879	-1.13	603.0778 (-H_2O), 585.0670 (-2H_2O), 559.0875, 519.0539, 477.0454 (-1PGU, -H_2O), 433.0565, 413.0302, 393.0224, 371.0398 (-2PGUs, +2), 339.0502, 309.0391, 283.0272, 245.0079 (-3PGUs, +2), 205.0492 (-3PGUs, -2H_2O, -2), 139.0029	1.32±0.18
9		9.423	212	745.1058	1.61	727.0949 (-H_2O), 701.1124, 659.0818, 579.0816, 537.0685, 477.0475 (-2PGU, -H_2O, +2), 411.0348, 355.0414, 311.0225 (-3PGU, -3H_2O, -2), 249.0416, 205.0158 (-4PGU, -2H_2O), 163.007	-
10	$C_{36}H_{26}O_{18}$	11.009	212, 272sh	745.1046	0.00	727.0935 (-H_2O), 665.0639, 619.0747 (-1PGU, -2H_2O, +2), 585.0676 (-1PGU, -2H_2O, +2), 559.0870, 517.0385, 477.0472 (-2PGU, -H_2O, +2), 441.0237, 389.0308, 353.0292, 309.0442, 231.0295, 205.0120 (-1PGU, -36), 177.0194, 139.0052	0.96±0.04
11		11.112	212	745.1049	0.40	727.0943 (-H_2O), 709.0858 (-2H_2O), 619.0735 (-1PGU), 583.0538 (-1PGU, -2 H_2O), 525.5692, 477.0478 (-2PGU, -18, +2), 443.0338, 371.0425, 339.0503, 263.0168, 229.0121, 203.0354 (-4PGU, -2H_2O, -2), 177.7941, 139.0033	
12		11.732	-	869.1238	3.57	851.1094 (-H_2O), 833.0920 (-2H_2O), 727.0922 (-1PGU, -H_2O, +2), 693.0811, 641.0496, 601.0574 (-2PGU, -H_2O, +2), 567.0528, 513.0421, 497.0697, 477.0392, 409.0238, 353.0272, 337.0401, 229.0136, 204.8425 (-5PGU, -2H_2O, +1), 139.0014	-
13	$C_{42}H_{30}O_{21}$	12.190	-	869.1198	-1.04	851.1086 (-H_2O), 775.0068, 744.0918 (-1PGU, +1), 689.0585, 619.0710 (-2PGU, +2), 583.0460 (-2PGU, -2H_2O, +2), 511.0480, 459.0351, 426.0419, 373.0522, 338.0352, 303.3885, 229.0143, 175.0369	-

Supplementary material - Figure S.1

Supplementary material - Figure legends S.1