Thesis for the Degree of Doctor of Philosophy

Enhancing the quality of seafood products through new preservation techniques and seaweed-based antioxidants:

Algal polyphenols as novel natural antioxidants

Tao Wang

Faculty of Food Science and Nutrition
School of Health Sciences
University of Iceland
Reykjavík, December 2009

Academic Dissertation

University of Iceland, Faculty of Food Science and Nutrition

In collaboration with

Matís (Icelandic Food Research),

United Nations University-Fisheries Training Programme, Reykjavik, Iceland

Supervised by

Dr. Guðrún Ólafsdóttir, School of Engineering and Natural Sciences, University of Iceland

Ph D Committee

M. Sc. Guðjón Þorkelsson, Matís (Icelandic Food Research) and School of Health Sciences, University of Iceland

Dr. Hörður G. Kristinsson, Matís (Icelandic Food Research) and Department of Food Science and Human Nutrition, University of Florida, USA

Dr. Charlotte Jacobsen, DTU Aqua, National Institute of Aquatic Resources, Technical University of Denmark, Denmark

Dr. Guðmundur Óli Hreggviðsson, Matís (Icelandic Food Research) and School of Engineering and Natural Sciences, University of Iceland

Opponents

Dr. Ingrid Undeland, Chalmers University of Technology, Sweden

Dr. Kristberg Kristbergsson, School of Health Sciences, University of Iceland

Dissertation for the Degree of Ph.D. in Food Science

Printed in Iceland by Leturprent, Reykjavik, 2009

ISBN 978-9979-9928-3-7

UNITED NATIONS UNIVERSITY
Fisheries Training Programme

ABSTRACT

Novel preservation techniques and addition of antioxidative and functional ingredients need to be implemented to maintain the prime quality and ensure the storage stability, to meet the demand of the global market for health-promoting functional seafood. The objective of this thesis was to investigate the effect of packaging and chilling preservation techniques on quality changes of fresh lean fish and study the potential application of algal polyphenols as novel natural antioxidants to prevent lipid oxidation of fish muscle and fish protein based products.

The key reasons for quality deterioration of perishable seafood products are bacterial growth and changes caused by oxidative and enzymatic reactions. Modified atmosphere packaging (MAP) in combination with superchilled storage was effective in retarding microbial growth and extending the prime quality shelf life of fresh cod loins. However, lipid oxidation may not be effectively inhibited and contributes to quality changes. The extraction and characterisation of antioxidant activity of algal polyphenols and their inhibitory effect on lipid oxidation in fish model systems was therefore the main focus of the studies. Among the ten species screened by *in vitro* antioxidant tests, *Fucus vesiculosus* were found to possess the highest total phlorotannin content (TPC) and the greatest radical scavenging activities. Subfractions rich in oligomeric and polymeric phlorotannins exhibited potent DPPH radical scavenging activity, comparable to or even higher than several commercial antioxidants. Successive ultra-filtration showed that phlorotannins in *F. vesiculosus* are mainly composed of high molecular weight polymers. But no clear correlations were observed between degree of polymerisation, molecular size and chemical antioxidant activities. The chemical antioxidant activities of *F. vesiculosus* extract/fractions did not adequately reflect their ability to prevent lipid oxidation in fish model systems. The oligomeric subfractions at 300 mg/kg were highly effective in inhibiting lipid oxidation while polymeric subfractions only had slight retarding effect. Additionally, all the subfractions separated by Sephadex LH-20 column and ultra-filtration showed potent *in vitro* ROS (Reactive Oxygen Species) scavenging ability in a mononuclear cell-based bioassay as well as high ACE (angiotensin converting enzyme) inhibitory activities, demonstrating the multiple bioactive properties of phlorotannins. The efficiency of enzyme-assisted extraction of algal polyphenols is species and enzyme-dependent. Protease treatment enhanced the extraction of polyphenols and other hydrophilic antioxidant components from *Palmaria palmata* but negative effects were observed for *F. vesiculosus* and *Laminaria hyperborea*. The studies provide first evidence on the inhibitory effects of phlorotannin-rich extract and fractions on lipid oxidation in fish model systems. Valuable information gained on antioxidant properties and possible health promoting effects of phlorotannins derived from marine algae, are not only of academic interest but also of great practical value for the food industry.

Keywords: Seaweeds; *Fucus vesiculosus*; phlorotannins; antioxidant; lipid oxidation; antioxidant assay; fish model systems; shelf-life; MAP; superchilled storage

ÁGRIP

Þörf er á nýjum aðferðum ásamt þróun á náttúrulegum andoxunarefnum til að auka geymsluþol unninna sjávarafurða og styrkja framleiðslu á hollu markfæði úr sjávarafurðum. Markmið verkefnisins var að rannsaka áhrif nýrra pökkunaraðferða og undirkælingar á gæði fersks fisks jafnframt því að einangra og rannsaka virkni fjölfenóla úr þörungum og nota sem nýstárleg náttúrleg andoxunarefni til að koma í veg fyrir oxun í fiski og unnum afurðum úr fiskpróteinum.

Aðalástæður gæðarýrnunar sjávarafurða er vegna örveruvaxtar og oxunar (þráa). Samvirkni loftskiptra pakkninga og undirkælingar reyndist árangursrík til að viðhalda ferskleika og auka geymsluþol þorskhnakka, en oxun fjölómettaðra fitu í fiski gæti einnig valdið gæðarýrnun við þessar aðstæður. Nýting á fjölfenólum úr þörungum sem náttúrleg andoxunarefni í fiskafurðir var aðalviðfangsefni rannsóknarinnar sem fólst í skoðun á virkni mismunandi þátta úr þörungum með *in vitro* andoxunarprófunum og virkni þeirra í fiskmódel kerfum. Rannsóknir á tíu tegundum af íslenskum þörungum sýndu hæsta innihald af fjölfenólum og háa andoxunarvirkni hjá tegundinni *Fucus vesiculosus* (ísl. bóluþang).

Grófhreinsaðir phlorotannin útdrættir úr *F. vesiculosus* höfðu framúrskarandi *in vitro* andoxunarvirkni, sem var sambærileg við eða meiri en tilbúin andoxunarefni. Frekari þáttun á phlorotanninum með örsíun sýndi að þættir með hærri mólþunga voru yfirgnæfandi í *F. vesiculosus*, en engin fylgni var á milli fjölliðunar, mólþunga og *in vitro* andoxunarvirkni. Einnig kom í ljós að andoxunarvirkni útdrátta og undirþátta af phlorotannin úr *F. vesiculosus,* sem var mæld með *in vitro* prófum, gaf ekki sambærilega mynd af virkni þeirra og prófanir í fiskkerfum. Undirþættir af fáliðu einingum (300 mg/kg) voru mjög áhrifaríkir til að hemja oxun, en fjölliðu þættir höfðu aðeins lítilsháttar virkni. Einnig var sýnt fram á í einkjörnunga kerfum, að mismunandi undirþættir höfðu hemjandi áhrif á virk oxunarefni (ROS, reactive oxygen species) auk blóðþrýstingslækkandi áhrifa (ACE, angiotensin converting enzyme próf). Ensím hvataður útdráttur með próteasa meðhöndlun jók nýtni fjölfenóla og annarra vatnssækinna andoxunarefna úr sölvum (*Palmaria palmata*), en reyndist ekki gagnlegur fyrir *F. vesiculosus* og *Laminaria hyperborea*. Rannsóknirnar sýndu fram á andoxunarvirkni þörunga og sannreyndu í fyrsta sinn hemjandi áhrif phlorotannin-ríkra útdrátta og undirþátta úr *F. vesiculosus* á lípíðoxun í fiskkerfum. Mikilvægar upplýsingar, sem fengist hafa í þessari rannsókn um andoxunareiginleika þörunga og jákvæða svörun í lífvirkni prófunum, gefa vísbendingar um hugsanleg heilsubætandi áhrif phlorotannina úr sjávarþörungum. Niðurstöðurnar eru ekki eingöngu vísindalega mikilvægar heldur er einnig möguleiki á hagnýtingu í matvælaiðnaði.

Lykilorð: Þörungar; *Fucus vesiculosus*; phlorotannins; andoxunarefni; lípíðoxun; andoxunarpróf; fiskkerfi; geymsluþol; MAP; undirkæling

LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, referred to in the text by their respective Roman numerals:

I. Wang, T., Sveinsdóttir, K., Magnússon, H., & Martinsdóttir, E. (2008). Combined application of modified atmosphere packaging and superchilled storage to extend the shelf life of fresh cod (*Gadus morhua*) loins. *Journal of Food Science*, 73 (1), S11–S19.

II. Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic Seaweeds. *Food Chemistry*, 116 (1), 240-248.

III. Wang, T., Jónsdóttir, R., Kristinsson, H. G., Hreggviðsson, G. O., Jónsson, J. O., Þorkelsson, G., & Ólafsdóttir, G. Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. Submitted.

IV. Wang, T., Jónsdóttir, R., González, M. J., Medina, I., Kristinsson, H. G., Raghavan, S., & Ólafsdóttir, G. Antioxidant properties of solvent extracts and fractions from the brown algae *Fucus vesiculosus*. Manuscript.

V. Wang, T., Jónsdóttir, R., Kristinsson, H. G., Þorkelsson, G., Jacobsen, C., Hamaguchi, P. Y., & Ólafsdóttir, G. Inhibition of haemoglobin-mediated lipid oxidation in washed cod muscle and cod protein isolates by *Fucus vesiculosus* extract and fractions. Submitted.

The introduction chapter of the thesis is partly based on the following publications:

Wang, T., Ólafsdóttir, G., Jónsdóttir, R., Kristinsson, H. G., & Johannsson, R. Functional and nutraceutical ingredients from marine macroalgae. In: C. Alasalvar, K. Miyashita, F. Shahidi, & U. Wanasundara (Eds.). *Seafood quality, safety, and health effects*. Oxford: Blackwell publishing limited. (in print).

Wang, T., Jonsdóttir, R., Ólafsdóttir, G., & Kristinsson, H. G. Antioxidant properties of marine macroalgae. In: H. G. Kristinsson, & S. Raghavan (Eds.). *Antioxidants and Functional Components in Aquatic Foods*. Ames, IA: Wiley-Blackwell, John Wiley & Sons, Inc. Submitted.

Abbreviations

ACE	Angiotensin I converting enzyme
ANOVA	Analysis of variance
ARP	Antiradical power
DPPH	2,2-diphenyl-1-picrylhydrazyl (radical scavenging assay)
GC-MS	Gas chromatography-mass spectrometry
GC-O	Gas chromatography-olfactometry
Hb	Haemoglobin
HMW	High molecular weight
HP	Haemeprotein
LMW	Low molecular weight
MAP	Modified atmosphere packaging
ORAC	Oxygen radical absorbance capacity
PCA	Principle component analysis
PGE	Phloroglucinol equivalents
PLSR	Partial least squares regression
PUFA	Polyunsaturated fatty acids
QDA	Quantitative descriptive analysis
ROS	Reactive oxygen species
TBARS	Thiobarbituric reactive substances
TES	Total extractable substances
TMA	Trimethylamine
TPC	Total phenolic content
TVB-N	Total volatile basic nitrogen
TVC	Total viable count

TABLE OF CONTENTS

AIMS

The overall aim of this study was to underpin the development of added value, health promoting functional seafood. This was achieved by investigation of the combined effects of packaging and superchilling on quality changes and shelf life of fresh lean fish and by exploring the potential application of algal polyphenols as novel natural antioxidants to control lipid oxidation and quality deterioration in fish muscle and fish protein based products. The study was divided into the following tasks:

- Evaluate the combined effect of modified atmosphere packaging and superchilled storage on shelf-life and quality changes of fresh cod loins.
- Screen for antioxidant activities in various types of Icelandic seaweeds to justify the selection of species with high antioxidant potentials for possible applications in fish products and functional food formulations.
- Evaluate the suitability of different commercial enzymes for enhanced extraction of water-soluble antioxidant ingredients from abundant red seaweed *Palmaria palmata*.
- Develop the extraction and separation methods and characterise the antioxidant properties of phlorotannin components from *F. vesiculosus* to gain a basic understanding of how molecular size and degree of polymerisation may influence the antioxidant activity.
- Investigate the contribution of phlorotannins to the overall antioxidant activity and how intrinsic antioxidant characteristics, molecular size may influence the antioxidant effect on hemoglobin-mediated lipid oxidation in fish model systems.

In order to demonstrate other possible health promoting effects of phlorotannins, an additional aim was to study their *in vitro* ROS scavenging and ACE inhibitory activities.

1 INTRODUCTION

In the search for effective natural antioxidants based on seaweed to improve the oxidative stability of seafood products, it is crucial to understand the complex mechanisms of lipid oxidation in fish muscle and the chemical and physical factors influencing the effectiveness of antioxidants. The knowledge on the chemical structure of algal polyphenols, the structure-antioxidant relationship and extraction methods is prerequisite for the development of seaweed based antioxidants. Because antioxidants behave differently in different antioxidant assay system, a better understanding on the chemistry behind the antioxidant tests and their merits and limitations is needed.

Herein the scientific background related to the PhD study is put into perspective by giving a general overview of lipid oxidation and natural antioxidants. Studies on algal polyphenols as novel natural antioxidants are reviewed and the papers of the thesis are put in context to clearly demonstrate how the PhD work is giving new evidence beyond the state of art.

1.1 Lipid oxidation in fish and fish products

Lipid oxidation is a major cause of quality deterioration in muscle foods during processing and storage. Due to high level of polyunsaturated fatty acids and low content of antioxidants, fish products are more susceptible to lipid oxidation than other muscle foods (Tichivangana and Morrissey 1985). The susceptibility of fish tissue to oxidation is dependent not only on total amount of lipids, but also on fatty acid composition and their location in fish muscle tissue. Lipid oxidation in fish is initiated in the intracellular phospholipid fractions of muscle cell membranes. Therefore, oxidative deterioration is a problem in both fatty and lean fish species. Lipid oxidation leads to the development of undesirable odours and flavours that limit the shelf-life of fish products and their utilisation in convenience seafood-based products. Moreover, the consequent decrease in nutritional values and generation of potentially toxic reaction products has become an increasing concern. Accumulated scientific evidence indicates that lipid oxidation products are involved in the etiology of atherosclerosis as well as in many other human pathologies such as inflammation, cancers, aging and Alzheimer's disease (Halliwell, 1997; Valko *et al*., 2007).

1.1.1 Mechanisms of lipid oxidation

Lipid oxidation in fish can be initiated, promoted and accelerated by a number of mechanisms. These include haemeprotein-mediated lipid oxidation; enzymatic and non-enzymatic generation of highly reactive, unstable radical oxygen species; the production of singlet oxygen and thermal- or iron-mediated homolytic cleavage of hydroperoxides (Kubow, 1992).

1.1.1.1 Haemeprotein-mediated lipid oxidation

Haemeproteins (HP) are the primary and endogenous catalysts of lipid oxidation in fish muscle. The redox state and source of HP directly influences their ability to initiate lipid oxidation. Oxidised HP (such as met-haemoglobin and met-myoglobin) and/or deoxygenated HP (deoxy-HP) have been reported to be more potent pro-oxidants than oxygenated HP at post mortem pH values (Richards and Hultin, 2000; Richards and Hultin, 2003; Undeland *et al*., 2003b; Undeland *et al*., 2004).

Deoxy-Hb is able to initiate lipid oxidation at low lipid hydroperoxide concentrations. Although the exact mechanism by which deoxy-Hb accelerates lipid oxidation is not fully understood, it acts as a potential initiator through a pathway that appears independent of lipid hydroperoxide (Richards and Hultin, 2000). The presence of deoxy-Hb can accelerate the rate of met-Hb formation compared to fully oxygenated Hb (Shikama & Matsuoka, 1986). DeoxyHb-stimulated met-Hb formation can be explained by the high spin state of ferrous heme iron in deoxy-Hb and easier access of reactive oxidants to the heme crevice (Richards *et al*., 2002). Lowering the pH within post-mortem pH causes increased deoxygenation of Hb (Manning *et al*., 1998; Richards *et al*., 2002).

It is well accepted that the oxidised forms of HP are the most active in catalysing lipid oxidation and breaking down lipid hydroperoxides into secondary oxidation products. The formation of met HP (met-Hb and met-Mb) is highly correlated with lipid oxidation in muscle foods. Baron *et al*. (1997) reported that met-Mb is an effective prooxidant at acidic pH and in the presence of preformed hydroperoxides.

During the autoxidation process of HP, oxygen is released from oxy-HP to form ferric (Fe^{3+}) met-Hb, met-Mb and superoxide anion radical via a proton or deoxygenerated HP mechanism.

$$\text{oxy}(+2)\text{HP} + H^+ \rightarrow \text{met}\,(+3)\text{HP} + \bullet\text{OOH}$$

$$\text{deoxy}(+2)\text{HP} + O_2 \rightarrow \text{met}(+3)\text{HP} + O_2\bullet^-$$

The superoxide anion radical can rapidly be converted into hydrogen peroxide.

$$2\ \text{oxy}(+2)\text{HP} \rightarrow 2\ \text{met}\,(+3)\text{HP} + 2O_2\bullet^-$$

$$2O_2\bullet^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The formation of met haemoproteins is critical to the onset of lipid oxidation since the ferric form HP can break down preformed lipid hydroperoxides to stimulate formation of compounds capable of initiating and propagating lipid oxidation (Everse and Hsia, 1997). In addition, Met-Hb and met-Mb are more readily to unfold and release their heme moiety compared to oxy-HP or deoxy-HP (Hargrove *et al*., 1996). The released hemin is the critical entity that drives HP-catalysed lipid oxidation in fish muscle.

Met HP further react with either liberated H_2O_2 or lipid hydroperoxides to form highly reactive ferryl Hb and ferryl Mb radicals. Hydrogen peroxide activation of met-Hb and met-Mb is a necessary step in the conversion of Met HP to pro-oxidants. Interaction between Met HP and H_2O_2 is a complex mechanism, resulting in the formation of the highly reactive perferryl derivatives (Grunwald and Richards, 2006). The prooxidative activity of ferrylMb was found to be independent of pH and of lipid concentration, therefore, ferrylMb can be highly effective prooxidant both under the conditions found in muscle food and under physiological conditions (Baron and Andersen, 2002).

1.1.1.2 Enzymic initiation systems

Several endogenous enzyme systems in fish tissue have been reported to be involved in the initiation of lipid peroxidation. Among them, lipoxygenases and peroxidases catalyse the formation of highly reactive hydroperoxides which can further propagate the lipid peroxidation chain reaction, especially under high storage temperature.

Lipoxygenase-catalysed lipid oxidation: Lipoxygenases (LOXs) catalyse the oxidation of polyunsaturated fatty acids containing a (Z,Z)-1,4-pentadiene moiety to produce their corresponding hydroperoxide fatty acids with *Z,E*-diene conjugation. In fish blood tissues, LOX activity in platelets is higher than that found in plasma or erythrocytes. The most active LOXs are in the gill and skin tissues, followed by brain, ovary, muscle, eye, liver, spleen, and heart, which has less than 10% of those found in gill and skin (Pan and Kuo, 2000).

Unlike lipid autoxidation, oxidation reactions catalysed by LOX are characterised by the common features of enzyme catalysis: substrate and conformation specificity, optimal activity under optimal conditions (temperature and pH) and high susceptibility to heat treatment. LOX isozymes have been shown to possess a high degree of substrate and conformation specificity. The number and position of pentadiene groups in the polyunsaturated fatty acid molecule have great impact on the end-products of the oxidation (Cadwallader, 2000; Hsieh and Kinsella, 1989).

NADH-dependent oxidase systems: In addition to LOXs, NADH-dependent oxidase systems have also been reported in fish muscle microsomes or fish tissue (Han and Liston, 1987; Soyer and Hultin, 2000). In muscle systems, both sarcoplasmic reticulum (SR) and mitochondria membranes have been demonstrated to generate active oxygen species in the presence of NAD(P)H and ferric iron. The difference between fish and other muscle systems is its significant activity at low refrigeration temperatures and at frozen temperatures. In microsomes from beef and chicken, NADPH is the favoured reducing agent over NADH, whereas in microsomes including SR of several marine fish species, the enzymic system utilises NADH preferentially (McDonald and Hultin, 1987).

1.1.1.3 Singlet oxygen generation systems

It is well known that the autoxidation of unsaturated lipids can be greatly catalysed or accelerated by exposure to light through direct photooxidation or photosensitised oxidation. Singlet oxygen ($^1O_2$) is highly reactive in photooxidation deterioration since it is more electrophilic than triplet state oxygen ($^3O_2$), reacting approximately 1500 times faster with methyl linoleate than normal triplet oxygen (Nawar, 1996). Hydroperoxides formed by singlet oxygen can further propagate the autoxidation of the unsaturated fatty acids. Lipid oxidation in seafoods can be initiated and promoted by the production of singlet oxygen. Compared with other food lipids, seafood lipids are particularly susceptible to photooxidation.

1.1.1.4 Effect of LOXs and haemoglobin on volatile profiles and odour characteristics

Because of the different mechanism and kinetic involved in LOX and HP-mediated lipid oxidation, the profiles of the produced volatile compounds and odour characteristics of the oxidised lipid can vary significantly. Fu *et al*. (2009) compared the effect of LOXs and Hb on the kinetics of lipid oxidation in silver carp (*Hypophthalmichthys molitrix*) mince model systems. The model containing LOXs produced distinctive fishy odour whereas strong oxidised oil dominated in model with added Hb. Hexanal, 1-penten-3-ol and 1-octen-3-ol were identified as the major odour active compounds developing during Hb induced oxidation while LOX system was more characterised by fishy odours contributed by *cis*-4-heptenal, heptanal and 2,4-heptadienal.

1.1.1.5 Cooperative actions of different pro-oxidative systems in fish muscle

The mechanisms of lipid oxidation in fish muscle is very complex, involving not only enzymic initiation systems but also oxidation initiated and promoted by other mechanisms

such as photosensitised and HP-catalysed oxidation. The rapid oxidation of fish lipids can be attributed to the cooperative actions of these pro-oxidative systems in fish muscle.

By rapid production of hydroperoxides postmortem, the lipoxygenase may act as an important initiator to activate the process of lipid autoxidation. It is also responsible for the rapid generation of fresh fish odours in some fish species (Josephson *et al*., 1984). However, it should be stressed that most volatile compounds (i.e. hexanal, cis-4-heptenal, 2,4-heptadienal and 2,4,7-decadienal) contributing to the characteristic painty, fishy and cod liver oil-like odours in stored fish and fish oils are produced via autoxidation of PUFA. The oxidised haemeproteins such as met-Hb and met-Mb are the most potent pro-oxidants and the major contributor to lipid oxidation in fish muscle. It was observed that the pro-oxidative activity of HP remained longer than that of LOXs in herring and sardine (Medina *et al*., 1999). This may be due to the instability of LOXs. The hydroperoxides generated during lipid oxidation may cause the oxidation or modification of sulphydryl groups of LOX, leading to the inactivation of LOXs at the later stage of oxidation. Similarly, rapid onset of lipid oxidation was found in minced carp model containing LOX, but it had lower PV and TBARS values at the later stage compared to Hb induced oxidation system (Fu *et al*., 2009).

1.1.1.6 The impact on the quality of fish and fish products

Lipid oxidation is one of the main mechanisms responsible for quality deterioration in fish and fish products, resulting in undesirable changes in aroma and flavour, texture, colour and nutritive value. In addition, primary lipid oxidation products (hydroperoxides) and secondary lipid oxidation products (aldehydes and ketones) can react with other food components such as proteins, vitamins. These interactions could either form complexes that limit the bioavailability of these nutrients, alter the functional properties or lead to the formation of toxic products.

Interactions between lipid oxidation products and proteins cause undesirable property changes of proteins including texture deterioration, decrease in protein solubility, change in colour and loss of nutritive value. Oxidised lipids induce remarkable conformation change of protein by altering the surface charges, increasing hydrophobicity, inducing unfolding and denaturation of protein molecules or forming protein-lipid complexes. Since functional properties of proteins are related to protein surface properties, conformational changes in the protein structure can significantly affect functional properties important to final products such as solubility, viscosity, water-holding capacity, gel-forming ability and emulsifying capacity (Xiong and Decker, 1995).

The complexation between lipid oxidation products and free amino acids or free amino groups of proteins result in the formation of yellow intermediary products such as imines, pyridine derivatives and aldolization products (Pokorny and Sakurai, 2002). These intermediary products can further polymerize into dark brown pigment with macromolecule. On the other hand, the basic groups in proteins can catalyze aldol condensation of carbonyls compounds produced from lipid oxidation, leading to the formation of brown pigments (Nawar, 1996).

Oxidative modification of proteins by hydroperoxides and secondary lipid oxidation products results in alteration of the nutritive value of proteins as manifested by marked reduction in the digestibility of protein as well as limitation of the amount and the degree of amino acid availability. Several essential amino acids such as lysine and methionine are highly susceptible to lipid oxidation products (Frankel, 1998).

1.2 Prevention of lipid oxidation by natural antioxidants

In the food industry, it is a common practice to use antioxidants as food additives to control lipid oxidation processes, retard the development of rancid off-flavours, improve colour stability and maintain the nutritional value of various fresh and further processed food products.

Many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroxyquinone (TBHQ) and propyl gallate have been widely used for more than 50 years. Because of the potential health hazards, their use as food additives is under strict regulation in many countries. Moreover, their antioxidant effectiveness is largely dependant upon the type of food systems. For example, BHT and α-tocopherol have been reported to be ineffective in retarding the oxidative deterioration in complex food system as fish muscle (He and Shahidi, 1997). The growing consumer preference for natural food products and the increased concern for the quality and safety in food industry have led to increased research on natural antioxidants.

1.2.1 Antioxidant effects in food systems

Great efforts are being devoted to the search for abundant, cheap and sustainable sources of natural antioxidants. Numerous studies have focused on natural antioxidants in terrestrial plants and their application in food systems to prevent oxidation. The antioxidant properties of various plant crude extracts such as those of rosemary, sage, oregano, clove, basil, green tea have been studied in different food systems. Agricultural and industrial by-products of

plant original are abundant and cheap sources of natural antioxidants (Balasundram *et al*., 2006).

Extensive studies have also been carried out on purified compounds extracted from vegetal biomass including polyphenols, alkaloids, carotenoids, terpenoids, sterols and tocopherols. Among these natural antioxidant compounds investigated, the most abundant and active one is polyphenols. Different types of polyphenolic compounds have been identified from grape seeds, grape pomace, citrus seeds and peels, apple pomace and potato peel waste. Previous studies have shown that plant-derived polyphenolic compounds are promising natural antioxidants for control of oxidative deterioration of various muscle foods such as red meat, poultry and fish. The addition of tea catechins at a level of 300 mg/kg was shown to exhibit extraordinary antioxidant property toward lipid oxidation of meat lipids (Tang *et al*., 2001). The antioxidative effect of tea catechins was better than that of α-tocopherol at the same concentration. Flavonol-enriched fraction derived from cranberry crude extract and white grape dietary fiber concentrate was also reported to provide excellent oxidative stability in an ice stored cod mince model system (Lee *et al*., 2006b; Sanchez-Alonso *et al*., 2007).

1.2.2 Synergistic and antagonistic effects of antioxidants

In food and biological systems, antioxidants are present in naturally occurring combinations and the interaction between different groups of antioxidants plays an important role for their overall effects. It has been well established that appropriate combinations of antioxidants are more effective in preventing lipid oxidation than single antioxidants.

Synergism between antioxidants may arise *via* several different mechanisms (Becker *et al*., 2004):

- Regeneration of highly active antioxidant in the system by other less effective antioxidants or oxidation retarders.
- Interaction of antioxidants with different polarities, i.e. different phase distributions.
- Interaction of antioxidants with different mechanisms of action.
- Synergies between antioxidants and substances without antioxidant activity.

Cooperative effects between α-tocopherol and different plant polyphenols is one of the most extensively studied antioxidant synergism. The synergistic effects have been tested in various model systems including homogenous solutions of peroxidating methyl linoleate, bulk oil, o/w emulsions, low density-liposomes and liposomal suspensions (Becker *et al*., 2007; Pedrielli and Skibsted, 2002; Roberts and Gordon, 2003). Results from these studies have shown that α-tocopherol could be regenerated from their oxidised forms by quercetin,

catechin and other plant polyphenols in different model systems via sparing effect. Polyphenols may also act as sacrificial antioxidants and are rapidly consumed during the scavenging of free radicals, protecting tocopherols against radicals generated in the water phase. The combinations of different mechanisms may be involved in the synergy between tocopherol and polyphenols where polyphenols act as a transition metal chelator and tocopherol as radical scavengers, thus protecting each other against reactive oxygen species (Altunkaya *et al*., 2009). In the heterogeneous systems, these synergistic effects may also be related to their solubility characteristics and distribution in different phases (Graversen *et al*., 2008).

Optimal blend of antioxidants has been demonstrated as an effective antioxidant strategy to enhance the oxidative stability and shelf life of muscle foods. It has been found that combinations of three antioxidants with different mechanisms of action was more effective in minimizing lipid oxidation in *n*-3 PUFA-fortified meat products and the effects was superior to the use of any single antioxidant alone (Lee *et al*., 2006a). In this cooperative antioxidant defence system, phenolic antioxidants in rosemary extract serve as radical scavengers, sodium citrate as transition metal chelator and sodium erythorbate as reductant (Figure 1). They function cooperatively to ensure maximisation of the oxidative stability through controlling several crucial stages of the oxidation pathway. Paiva-Martins *et al*. (2003) reported a synergistic effect among olive oil phenolic compounds, α-tocopherol and ascorbic acid in the liposome systems. Olive oil phenols mainly act as scavengers against aqueous peroxyl radicals near the membrane surface and recycle α-tocopherol. α-tocopherol further assists in the defence against aqueous peroxyl radicals as well as scavenges the chain propagating lipid peroxyl radicals within membranes. They also suggested a possible role of ascorbic acid in helping scavenge aqueous radicals and regenerating olive polyphenols and α-tocopherol from their oxidised forms in the liposomal suspension. Strong synergistic antioxidant effects were also observed between bovine serum albumin (BSA) and several phenolic antioxidants in another study (Almajano and Gordon, 2004). The presence of BSA in sunflower oil-in-water emulsions led to a marked increase in antioxidant activity of epigallocatechin gallate (EGCG), caffeic acid and Trolox, although BSA itself did not show any significant antioxidant activity. Synergy between these water-soluble antioxidants and BSA could be explained by the complexation of these phenolic compounds with BSA via hydrophobic interactions and hydrogen bonding. Due to the surface active nature of the protein, the formed BSA-antioxidant adduct could be concentrated at the oil–water interface where the oxidation is initiated and exert their antioxidant effects towards lipid oxidation.

However, other studies revealed that α-tocopherol exerted a strong antagonistic effect with different polyphenol-rich plant extracts in lard and sunflower oil (Banias *et al*., 1992; Hras *et al*., 2000). A clear negative interaction was observed between α-tocopherol and certain phenolic acids (rosmarinic and caffeic acids) during AAPH-induced oxidation of linoleic acid in an aqueous dispersed system (Peyrat-Maillard *et al*., 2003). Becker *et al*. (2007) reported that the combination of α-tocopherol and quercetin produced an antagonistic effect in purified high olein sunflower oil.

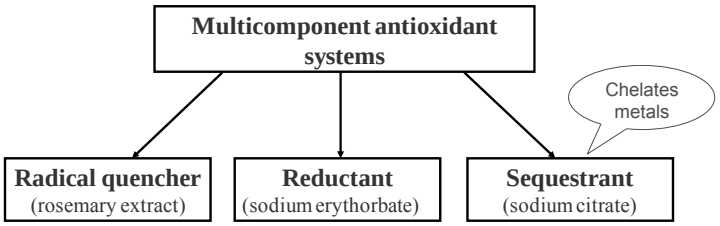


Figure 1. Cooperative antioxidant defence system consisting of rosemary extract, sodium citrate and sodium erythorbate (Lee *et al*., 2006a).

These studies clearly show that the synergism and antagonism of antioxidant combination is system-dependent. A multitude of factors can influence the synergistic or antagonistic effects in different model systems: the chemical structure of antioxidant molecules and the possible formation of stable intermolecular complexes; structural organisation of the lipid; polarity, solubility and other physical properties of the antioxidants; microenvironment of the reaction medium, especially the concentration of available antioxidants at the site of oxidation (Peyrat-Maillard *et al*., 2003; Becker *et al*., 2007).

1.2.3 Limitations and safety issues

It must be stressed however, that natural antioxidants also have various drawbacks that limit their large-scale application in food industry. The antioxidant performance of natural compounds is generally less effective than the synthetic antioxidants and larger amounts of natural antioxidants need to be added to food to achieve the same effect. The actual antioxidant effectiveness depends to a large extent upon food composition and particular processing and storage conditions. Plant-derived polyphenols are known to be prone to complex with proteins. The formation of covalently bound polyphenol-protein complexes may have negative impact on the antioxidant capacity of polyphenols as well as *in vivo* bioavailability of both proteins and polyphenols (Shahidi and Naczk, 2004). Many of the phenolic-rich extracts have intense characteristic odour, bitter taste and distinct colour, which adversely affect the sensory properties of food products (Gomez-Guillen and Montero, 2007).

Natural antioxidants have been generally considered safe for human consumption and the potential risk to human health has not been adequately investigated (Frankel, 2007). There is increasing evidence to suggest that caution should be exercised when assessing the safety of natural antioxidants. The complex chemical composition of plant extracts makes isolation and identification of compounds with the desired antioxidant activity a difficult task. In many studies, only the crude plant extract was used for evaluation of antioxidant activities which contain variable amounts of identified and unidentified compounds. Well-designed and well-controlled toxicological *in vitro* or *in vivo* cell or animal studies are recommended to evaluate the potential risk of toxicity of crude natural antioxidant extracts (Kruger and Mann, 2003).

1.3 Algal polyphenols as novel natural antioxidants

1.3.1 Occurrence and chemical structure

The antioxidant activities of different classes of polyphenolic compounds derived from terrestrial plants are well documented. Marine macroalgae are also gaining interest as a potential source of polyphenols. A series of polyphenolic compounds such as catechins (e.g. gallocatechin, epicatechin and catechin gallate), flavonols and flavonol glycosides have been identified from methanol extracts of several red and brown algae (Santoso *et al*., 2002; Yoshie *et al*., 2000; Yoshie-Stark *et al*., 2003). Phlorotannins, the dominant polyphenolic secondary metabolites only found in brown algae (Phaeophyta), have recently attracted considerable research interest because of their diverse biological activities. They have been identified from several brown algal families such as Alariaceae, Fucaceae and Sargassaceae.

Phlorotannins are mainly stored within cell organelles, called physodes, which are round to elliptical, vesicle-like, strongly refractive bodies observed in the cytoplasm of brown algae (Ragan and Glombitza, 1986; Schoenwaelder 2002). Moreover, it has been reported that phlorotannins tend to be concentrated within the outer cortical layers as well as the mitotic meristematic and meiotic sporogenous tissues in some species of brown algae (Ragan and Glombitza, 1986).

Phlorotannin concentrations vary dramatically among different taxonomic groups as well as among different geographical areas. The concentrations of phlorotannins in marine brown algae have been reported to range from undetectable to over 20% of algal dry mass (DM) (Ragan and Glombitza 1986). Intertidal fucoids generally contain higher amounts of phlorotannins than subtidal kelp species and the highest levels are found in Fucales and Dictyotales (Ragan and Glombitza 1986; Van Alstyne *et al*., 1999). Our study also

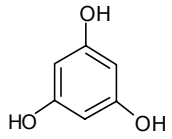
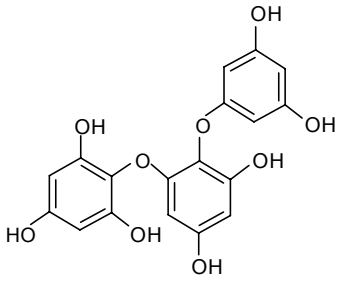
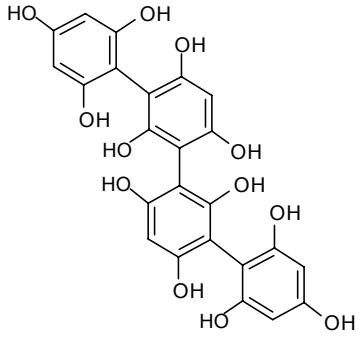
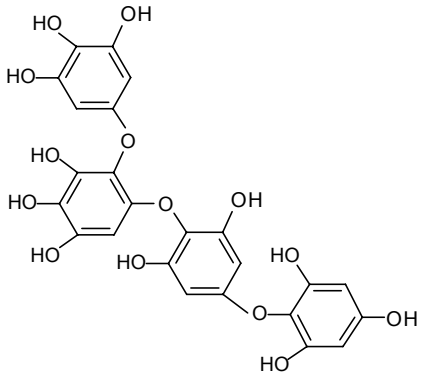
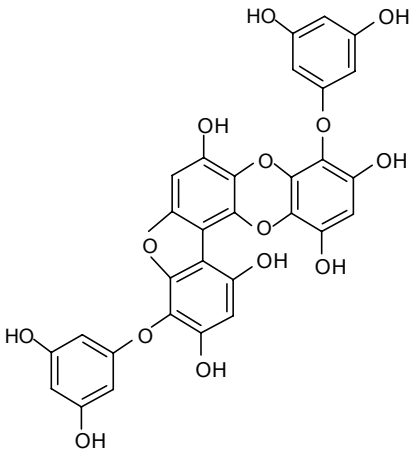
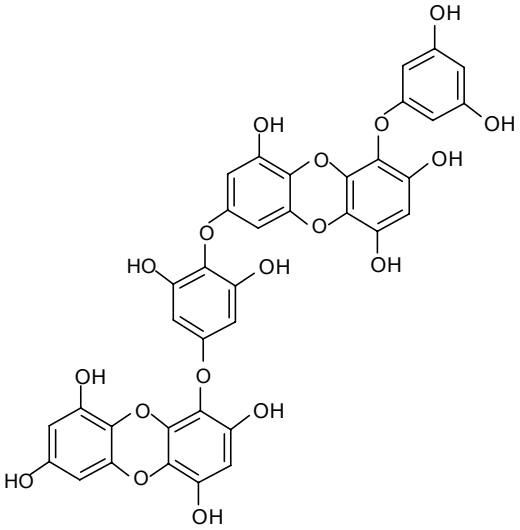
demonstrated significantly higher levels of total phlorotannin content (TPC) in three fucoid seaweeds (*F. vesiculosus*, *F. serratus* and *A. nodosum*) found on Icelandic seashores, whereas *L. digitata* contained very small amount of TPC (Wang *et al*., II).

Previous studies have shown that there are often significant intraspecific and intraplant variations in TPC of brown algae. The variations appear to be correlated with plant size, age, tissue type as well as environmental factors such as salinity, nutrient and light availability, season and water depth (Pavia and Toth, 2000). Biological factors such as herbivore grazing intensity and mechanical wounding have also been found to have some effects. Hemmi *et al*. (2005) investigated the phlorotannin allocation patterns among different tissue types of *F. vesiculosus* and detected significant within-plant difference. The lowest concentration was found in the receptacles and the highest concentration in the basal parts. However, no significant relationship was found between the apical longitudinal growth and phlorotannin content in the receptacles, or in apical and basal parts. Seasonal variation in phlorotannin levels was reported for *F. vesiculosus*, higher TPC levels generally occurred in summer and early autumn whereas lower amounts were observed during winter (Connan *et al.*, 2004). Moreover, sun exposed *F. vesiculosus* and *A. nodosum* plants were found to have substantially higher TPC than their shaded counterparts, supporting the important photo-protective roles of phlorotannins (Pavia & Toth, 2000).

Natural phlorotannins are oligomers and polymers of phloroglucinol (1,3,5-trihydroxybenzene) rather than monomers. The molecular weight of phlorotannins ranges from 126 Da to 650 kDa. It has been suggested that the structural types of phlorotannins can differ dramatically at different developmental stages. In young thallus, they may occur in algal cells in the form of fairly short oligomers. These oligomers may convert into longer and more complex phlorotannin polymers during the growth and development of the thallus (Koivikko *et al*., 2005).

Based on the type of structural linkages between the phloroglucinol sub-units (diphenylethers or biphenyls) and on the number of additional hydroxyl groups, phlorotannins can be systematically classified into six major subclasses: phlorethols, fucols, fuhalols, fucophlorethols, isofuhalols, and eckols (Ragan and Glombitza, 1986; Targett and Arnold, 1998). The chemical structures of common brown algal phlorotannins are presented in Table 1.

Table 1. Chemical structures of common brown algal phlorotannins.

	
Phloroglucinol	Triphlorethol A
	
Tetrafucol A	Tetrafuhalol A
	
Phlorofucofuroeckol	Dieckol

Fucols are phlorotannin polymers in which the phloroglucinol units are connected only by C-C (aryl-aryl) bonds and possess linear, angled and branched structures. The phloroglucinol units in phlorethols are linked only by C-O-C (aryl-ether) bonds. Fucophlorethols contain both biaryl and aryl-ether linkages. Fuhalols are connected exclusively via ether bonds in a regular sequence of para- and ortho-arranged ether bridges. Eckols are characterised by the occurrence of at least one three-ring moiety with a dibenzo-1,4-dioxin unit substituted by a

phenoxyl group at the C-4 position. They are usually of low molecular size and have been isolated only from some specific genera of brown algae including *Ecklonia*, *Eisenia* and *Alarieae*. Isofuhalols and endofucophlorethols are small, specialized groups isolated from specific algal genera such as *Chorda filum*. Moreover, some phlorotannins can also be sulphated or halogenated (Glombitza and Pauli, 2003; Ragan and Glombitza, 1986).

1.3.2 Antioxidant properties of algal polyphenols

1.3.2.1 In vitro antioxidant properties

Different species of seaweed display varying degrees of antioxidant activity. Brown algae generally contain higher levels of TPC than green and red algae and possess better antioxidant capacities. In particular, several species of brown seaweeds such as *Ecklonia cava*, *Ecklonia kurome, Fucus vesiculosus, Fucus serratus, Fucus spiralis, Hizikia fusiformis* and *Sargassum ringgoldianum* have been reported to exhibit superior antioxidant activity *in vitro*, which is well correlated with their content of TPC (Cerantola *et al*., 2006; Karawita *et al*., 2005; Nakai *et al*. 2006; Shibata *et al.*, 2008).

In a survey on 25 common seaweed species from the Japanese coast, fifty percent ethanol extract of *S. ringgoldianum* was found to have the highest radical scavenging activity (Nakai *et al*., 2006). The partially purified phlorotannin-rich fraction exhibited potent superoxide anion scavenging activity, which was around five times higher than that of catechin.

More recently, several phlorotannin derivatives including eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were isolated and identified from the Japanese Laminariaceous brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* and their antioxidant properties were evaluated by various antioxidant assays, including DPPH and superoxide anion radical scavenging activities as well as inhibition of phospholipid peroxidation in the liposome system (Shibata *et al.*, 2008). The scavenging activities of these phlorotannin compounds against DPPH and superoxide anion radicals were found to be around 2 to 10 times higher than those of catechin, α-tocopherol and ascorbic acid. These phlorotannin oligomers also had significant protective effects against peroxidation of phospholipid liposomes.

Many studies have shown that phenolic compounds are the main contributors to the antioxidant activity of various seaweeds. A positive correlation between TPC and antioxidant activity of different seaweed extracts has been well documented. Nagai and Yukimoto (2003) observed that the TPC of various seaweed extracts were positively correlated with both their antioxidant activity in a linoleic acid model system and the scavenging capacities against

DPPH and superoxide anion radicals. High degree of correlation between TPC and DPPH radical scavenging activity has also been reported for different solvent extracts ($R^2 = 0.971$) (Siriwardhana *et al*., 2003) as well as different organic and aqueous fractions of *H. fusiformis* ($R^2 = 0.921$) (Karawita *et al*., 2005). However, it should be pointed out that several other studies have also found poor or low correlation coefficients between TPC and antioxidant activity of some seaweed extracts. Other active compounds such as fucoxanthin and sterols in solvent extracts and sulphated polysaccharides, proteins or peptides, ascorbic acid, glutathione and mycosporine-like amino acids (MAA) in water and enzymatic extracts may also contribute partially to the overall antioxidant activity.

1.3.2.2 In vivo antioxidant activities

Although algal polyphenols, especially phlorotannins isolated from several brown algae have been reported to possess extraordinary antioxidant activity *in vitro*, their possible antioxidant effects in biological systems are not entirely convincing, mainly because of lack of reliable *in vivo* data. A few recent studies have shed some light on the antioxidant properties of algal polyphenols *in vivo* and their potential application in pharmaceutical and nutraceutical fields.

The antidiabetic and antioxidant effects of methanol extract from brown alga *Ecklonia stolonifera* (MEE) were recently studied in KK- A^y diabetic model mice (Iwai *et al*., 2008). Continuous administration of diets containing MEE for 4 weeks inhibited lipid peroxidation in plasma, red blood cells, liver and kidney of unfasted KK-A^y mice in a dose-dependent manner. The formation of plasma thiobarbituric acid-reactive species (TBARS) was almost completely inhibited in both low and high MEE diet groups and the levels were significantly lower than those in control group. The potent antioxidant activity of MEE appeared to have beneficial effect for the prevention of diabetic oxidative stress and related complications. The effective components in MEE were identified as phlorotannin compounds.

1.3.3 Antioxidant mechanism

The precise mechanisms underlying the antioxidant effects of algal polyphenols have not been fully elucidated. The putative mechanisms include free radical scavenging, singlet oxygen quenching, transition metal ion chelating and reducing capacities.

The potent free radical scavenging activities of plant and algae-derived phenolic compounds have been well documented and extensively reviewed (Nakai *et al.*, 2006; Rice-Evans *et al.*, 1997; Shahidi and Naczk, 2004a). Polyphenols have been found to possess the ability to capture free radicals, particularly peroxy radicals, which are the key reactive

intermediates/chain propagators, thereby terminating the chain reaction (Shahidi and Naczk, 2004a). Phenolic compounds can easily transfer a hydrogen atom to lipid peroxyl radicals, resulting in the formation of a stable phenoxyl radical and less reactive *cis, trans*-lipid hydroperoxide

$$LOO\bullet + ArOH \rightarrow LOOH + ArO\bullet$$

On the other hand, it has also been suggested that phenolic compounds can deactivate lipid peroxyl radicals by single electron transfer (Ou *et al.*, 2002)

$$LOO\bullet + ArOH \rightarrow LOO^{-} + ArOH^{+\bullet}$$

$$LOO^{-} + ArOH^{+\bullet} \rightarrow LOOH + ArO\bullet$$

The less reactive aroxyl radicals (ArO•) are obtained during this reaction, as their structures are stabilised through electron delocalisation. The aroxyl radicals may also react with lipid peroxyl radicals and convert them to more stable products with quinone structures, which does not initiate or propagate further oxidation of lipids (Canadanovic-Brunet *et al*., 2006; Rice-Evans *et al.*, 1997).

However, there are contradictory reports in the literature regarding metal chelating capacities of polyphenols. Some studies have shown that polyphenols derived from different plants as well as brown algae are potent ferrous ion chelators (Chew *et al.*, 2008; Senevirathne *et al*., 2006) and metal chelating ability are dependent upon their unique phenolic structure and the number and location of the hydroxyl groups (Santoso *et al*., 2004). In contrast, other authors have reported that metal chelation played a minor role in the overall antioxidant activities of several plant-derived phenolic compounds (Rice-Evans *et al*., 1996). In agreement with this finding, our study on ten species of Icelandic seaweeds showed that ferrous ion-chelating ability of seaweed extracts correlated neither with TPC, nor with DPPH radical scavenging activity or ORAC (Wang *et al*., II). Phlorotannin-rich solvent extracts and fractions were less effective in chelating ferrous ion than crude water extracts (Wang *et al*., II; Wang *et al*., IV). Other algal constituents such as polysaccharides, proteins or peptides may be more effective chelators than phlorotannins.

Reducing power has been suggested as an important indicator of the overall antioxidant capability of phenolic antioxidants. In general, the reducing properties are associated with the presence of reductones, which exhibit their antioxidant activities through the action of breaking the free radical chain by the donation of a hydrogen atom (Gordon, 1990). Reductones are believed not only to react directly with peroxides but also to prevent peroxide formation via reacting with certain precursors of peroxide. Algal polyphenols may act in a similar way as reductones since they possess intrinsic ability to donate electrons and react

with free radicals, converting them to more stable end product and block free radical chain reaction. Due to a common underlaying mechanism (electron/hydrogen donation), there is a fairly good linear correlation between TPC, the reducing activity and DPPH radical-scavenging activity (Duan *et al*., 2006; Wang *et al*., IV).

1.3.4 Structure-antioxidant activity relationship

It is believed that the strong antioxidant activity of brown algal phlorotannins is attributed to their unique molecular skeleton (Ahn *et al*., 2007). The antioxidant potency of polyphenols is highly related to phenol rings which act as electron traps to scavenge peroxy, superoxide-anions and hydroxyl radicals. Phlorotannins from brown algae have up to eight interconnected rings. They are therefore more potent free radical scavenger than other polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings (Hemat, 2007).

Unlike plant-derived polyphenols, the relationship between molecular structure and antioxidant activity of phlorotannins has been poorly investigated to date. Several lines of evidence indicate that oligomeric and polymeric phlorotannins are more potent antioxidants than the monomer phloroglucinol. Oligomerisation of phloroglucinol appears to be crucial for the antioxidant activity. Nakamura *et al*. (1996) observed that the fractions containing phlorotannin oligomers of higher MW such as dieckol, phlorofucofuroeckol and 8-8′ bieckol were more effective to extend the induction time of autoxidation of methyl α-linolenate than LMW fractions containing phloroglucinol and eckol. However, research aimed at clarifying the correlation between the antioxidant capacity and specific structural features of phlorotannins has yielded contradictory findings. Cerantola *et al*. (2006) isolated two types of structurally distinct phlorotannins from *Fucus spiralis* extracts and compared their DPPH radical scavenging activity. The first polymer had relatively simple structure and belonged to the fucol subclass whereas the second one was more complex and linked through aryl-aryl and aryl-ether bonds, thus belonged to the fucophlorethol subclass. Both polymeric phlorotannins showed equally high scavenging activity in comparison to ascorbic acid and phloroglucinol monomer, indicating that there was no clear association between the type of structural linkages and radical scavenging activity of phlorotannins. On the contrary, several other studies showed that the presence of O-bridge linkages (ether linkages) and the number and arrangement of phenolic hydroxyl groups in phlorotannin skeleton have profound influence on the antioxidant property. Phlorotannin compounds extracted from Ecklonia species such as *E. cava* and *E. stolonifera*, harvested from Jeju island of Korea, have been

reported to possess extraordinary antioxidant potential than the other species collected from the same area (Shin *et al*., 2006). The phlorotannins of *E. cava* and *E. stolonifera* belong to a unique polyphenolic category, characterised by a dibenzo-1,4-dioxin unit in the molecular skeleton. The unique structure appears to be restricted to some specific brown algae such as Eisenia and Ecklonia species and may be responsible for their potent antioxidant activities compared with the other brown algae. Moreover, several phlorotannin oligomers isolated from these species, including eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol were found to possess varying degrees of scavenging activities against DPPH and superoxide anion radicals (Shibata *et al*., 2008). The differential radical scavenging properties of these phlorotannin polymers were assumed to be related to the number of hydroxyl groups attached to the eckol skeleton.

1.3.5 Extraction methods

1.3.5.1 Organic solvent extraction

Solvent extraction is one of the most frequently used methods for the recovery of polyphenols from plants and marine macroalgae. Both the extractant type and solvent polarity are important factors that influence the extraction efficiency. Extraction of various classes of phenolics from different plant and algal material requires the development of different solvent systems with appropriate polarities. Phenolic compounds are generally more soluble in polar organic solvents than in water. The effective extractants recommended are aqueous mixtures of methanol, ethanol and acetone (Waterman and Mole, 1994).

Koivikko *et al*. (2005) compared the extraction efficacy of soluble phlorotannins from *F. vesiculosus* by using eight extractants with different polarities. 70% aqueous acetone was found to be the most efficient solvent. Acetone has been assumed to possess the ability to inhibit protein–polyphenol complex formation during extraction (Hagerman, 1988) or even break down hydrogen bonds formed between phenolic group and protein carboxyl group (Kallithraka *et al*., 1995). Therefore, higher extraction efficiency can be achieved by using this solvent.

In addition to extraction efficiency, the antioxidant activities of seaweed extracts are highly dependent on the types of solvent used. Change in extractant polarity alters its efficacy to extract a specific group of antioxidant compounds and influences the antioxidant properties of the extracts. In a study carried out by Anggadiredja *et al*. (1997), the methanol extract of *Sargassum polycystum* was found to exhibit higher antioxidant potency (evaluated by the

thiocyanate method) than the diethylether and *n*-hexane extracts, whereas the *n*-hexane extract of *Laurencia obtusa* was more effective than the diethylether and methanol extracts.

1.3.5.2 Enzyme-assisted aqueous extraction

Currently, organic solvent extraction is still widely used for the extraction of polyphenolic compounds from marine algae. Because of the increased public awareness and the growing environmental and safety concerns of using large amounts of some organic solvents, there is a great need to develop new extraction techniques. Moreover, the structural complexity and rigidity of the algal cell wall hinders the effective extraction and recovery of the intracellular bioactive constituents. As one of the promising techniques, enzyme-assisted aqueous extraction of polyphenols and other antioxidant ingredients from algal biomass offers several advantages over conventional water and solvent extraction methods. This technique allowed for simple and efficient production of natural antioxidants from seaweeds on an industrial scale (Heo *et al*., 2005). It does not use any organic solvent or other toxic chemicals during extraction. Improved extraction efficiency of phenolic compounds and higher antioxidant activity have been reported for enzymatic extracts from several brown algae, as compared with water and organic extract counterparts (Heo *et al*., 2005; Athukorala *et al*., 2006).

The proposed mechanism of enzymatic extraction is that cell wall-degrading enzymes can help to soften and macerate the algal tissue, weaken or disrupt the cell wall structure, break down complex interior storage materials, therefore facilitating the release of the intracellular polyphenols and other bioactive compounds (Siriwardhana *et al*., 2008). Furthermore, the breakdown/releasing of HMW polysaccharides and proteins may contribute to enhanced antioxidant activities.

The composition and antioxidant properties of enzymatic extracts from seaweeds are strongly dependant on seaweed species and specificity of the enzyme employed. In a study by Heo *et al*. (2005), seven species of brown algae were enzymatically hydrolysed by different types of commercial carbohydrases and proteases. Considerable differences in DPPH radical scavenging activity were observed among different seaweed species as well as different enzymatic treatments. Enzymatic extracts obtained from *E. cava* generally possessed higher DPPH radical scavenging capacity in comparison with other seaweed species. Particularly, Celluclast (carbohydrase) extract and Protamex (protease) extract exhibited approximately 70% scavenging activity at concentrations of 2 mg/ml, which were even higher than that of the synthetic antioxidant BHT at the same concentration. On the other hand, some enzymatic

extracts of *E. cava* and *S. coreanum* (especially Alcalase extract) exhibited rather weak DPPH scavenging activity.

1.3.5.3 New processes based on the use of sub- and supercritical fluids

In recent decades, several highly efficient and environmentally friendly techniques have been developed for the extraction of natural antioxidants from different vegetal biomass, including supercritical fluid extraction (SFE), subcritical water extraction (SWE), accelerated solvent extraction (ASE) and pressurized fluid extraction (PFE). The advantages of these extraction techniques include high selectivity, higher extraction efficiency, shorter extraction times, low operating temperature, reduction or elimination of the use of toxic and hazardous solvents and the generation of waste solvent.

Among these new technologies, SFE (especially using CO2 as extraction fluid) has been considered as one of the most promising techniques for the extraction of natural antioxidants suitable for use in food, cosmetic or pharmaceutical industry. The most intensely studied antioxidants extracted from plant materials by supercritical carbon dioxide (SC-CO_2) are phenolics, carotenoids, terpenoids, alkaloids and tocopherols. Supercritical or subcritical CO_2 extraction coupled with ethanol and/or methanol as a modifier has been successfully applied to extract polyphenols from grape seeds, olive leaves, rosemary leaves, pistachio hulls, wine industry by-products as well as apple and grape pomaces. SC-CO_2 (coulped with ethanol as co-solvent) was found to yield higher polyphenol recoveries from white grape pomace than traditional solid–liquid extraction (Pinelo *et al*., 2007). SC-CO_2 extracts also exhibited higher DPPH radical scavenging activity. However, little research has been conducted to examine the potential of sub- and supercritical CO_2 and other novel extraction techniques for the extraction of polyphenols from seaweeds.

1.3.6 Safety issues and health aspects

In order to direct seaweed based antioxidants into different food products or supplements for human consumption, the extraction and purification techniques is of prime importance. Up to now, organic solvents are still widely used in the extraction and purification of polyphenols from plant and marine algae. The extraction solvent must be adequately selected to meet the requirements set by EU regulations. According to the Annex I of the Directive 2009/32/EC of the European Parliament and of the Council on the approximation of the laws of the Member States on extraction solvents used in the production of foodstuffs and food ingredients (Recast), ethanol, ethyl acetate, propane, butane, acetone, carbon dioxide, nitrous oxide are

the solvents authorised to be used in compliance with good manufacturing practice (GMP) for all uses. Hexane, methyl acetate, ethylmethylketone, dichloromethane, methanol and propan-2-ol are the solvents authorised to be used under specified conditions with different maximum residue limits in the extracted foodstuff or food ingredient. In addition to solvent extraction, the extraction efficiency of other environmentally friendly technologies such as supercritical fluid extraction, subcritical water extraction, enzyme-assisted extraction and membrane separation system need to be investigated.

Polyphenol-rich crude extracts from edible seaweeds have been generally considered safe for human consumption. Recent toxicity studies further proved that oral administration of crude seaweed extracts and partially purified phlorotannins did not cause any toxic effects in rats or mice. A toxicity test was carried out to examine possible adverse effects of crude phlorotannins from brown algae, *Ecklonia kurome*. Results showed that there was no adverse effect in mice at level corresponding to a human dose of 90.0 g/60 kg per day in males and 64.3 g/50 kg per day in females (Nagayama *et al*., 2002). The crude ethanol extracts from *F. vesiculosus* didn't show any relevant toxic effects following a four week daily treatment in rats (Zaragozá *et al*., 2008). However, more toxicological *in vitro* or *in vivo* cell or animal studies are required to get supportive evidence (Kruger and Mann, 2003).

Recent studies have revealed that algal polyphenols, especially phlorotannins derived from brown algae not only exhibit potent antioxidant activity but also possess many other bioactive properties, including anti-inflammatory, anti-allergic, anti-tumour, anti-diabetic, antibacterial, HIV-1 reverse transcriptase and protease inhibitory activities (Plaza *et al*., 2008). In order to direct algal polyphenols into commercial applications as health-promoting natural antioxidants or functional food ingredients, substantial scientific evidence is needed (Codex Alimentarius, 2004; IFT Expert Report, 2005). Well-designed and well-controlled human intervention studies need to be carried out to systematically evaluate the possible health-promoting effects of new ingredients like algal polyphenols, to meet the requirements of the new legislations of health claims for foods (Asp and Bryngelsson, 2008; Regulation (EC) No. 1924/2006).

1.4 Methods to evaluate the antioxidant capacity *in vitro* and in food systems

1.4.1 Merits and limitations of various *in vitro* antioxidant assays

A wide variety of *in vitro* chemical tests have been developed to evaluate the antioxidant capacity of natural antioxidants of different origin. The most widely employed antioxidant

tests are those measuring the free radical scavenging capacity, the uptake of oxygen, the inhibition of induced lipid autoxidation, the chelation of the transition metals and the reducing power (Decker *et al*., 2005; Prior *et al*., 2005).

There is no universal antioxidant which is highly effective in all food systems and antioxidants behave differently in media with different polarities and phase states. And also no single one-dimensional assay is sufficient to identify the possible mechanisms involved in the antioxidant reactions (Frankel and Meyer, 2000). When selecting appropriate antioxidant methods to assess the antioxidant activity of potential antioxidant, the primary factors to consider are the possible mechanisms of antioxidant action and the relevance of these actions to what might occur in the target substrate. With regard to the initial screening experiment, the use of multiple *in vitro* tests based on different mechanisms is necessary to gain a more comprehensive antioxidant profile. It should also be noted that the reaction conditions, substrate and antioxidant concentration used in different antioxidant assays may have a great influence on the antioxidant effectiveness. Analysis conditions, substrate, and antioxidant concentration should simulate real food or biological systems as closely as possible (Frankel, 2007; Frankel and Meyer, 2000).

Huang *et al*. (2005) and Prior *et al*. (2005) summarise the chemical principles of various antioxidant capacity assays and established a classification depending upon the chemical reactions involved: (1) assays based on hydrogen atom transfer (HAT) reaction and (2) assays based on single electron transfer (ET) reaction.

1.4.1.1 HAT methods

Most HAT assays are kinetics-based, and use a competitive reaction scheme in which antioxidant and substrate kinetically compete for peroxyl radicals thermally generated through the decomposition of azo compounds. Among the methods based on HAT, the most frequently employed are oxygen radical absorbance capacity (ORAC) and total radical trapping antioxidant parameter (TRAP).

The HAT mechanisms of antioxidant action can be summarised by the reaction:

$$ROO\cdot + AH/ArOH \rightarrow ROOH + A\cdot/ArO\cdot$$

Oxygen radical absorbance capacity (ORAC): ORAC assay has been increasingly employed by academics and the food industry as a method of choice to evaluate the antioxidant capacities of foods. The validity and evaluation of this assay has been supported by the U.S. Department of Agriculture. In 2007, an updated database was released as part of

the USDA nutrient database where list the ORAC value for 277 commonly consumed foods in the United States.

The ORAC assay has several advantages over other antioxidant capacity assays. ORAC measure the radical scavenging activity against the peroxyl radical, which is the most prevalent free radical in both food and biological systems. The reaction is conducted under physiological conditions and therefore is believed to be more biologically relevant. The AUC approach allows the quantification of both the time course and degree of inhibition (Huang *et al*., 2005). A modified ORAC assay can measure the antioxidant capacity of both hydrophilic and lipophilic antioxidant components in a given sample (Huang *et al*., 2002). It can also be adapted to determine the antioxidant action against other reactive oxygen species.

However, ORAC assay is relatively complicated and time consuming. ORAC involves the complex reaction of azo compounds such as AAPH to generate peroxyl radicals. This reaction is highly sensitive to temperature variations and may lead to great intra-assay variability and diminished run-to-run reproducibility even when performed under temperature controlled conditions. The reference standard Trolox is not structurally related to any phenolic compounds exhibiting antioxidant activity in foods (Frankel, 2007). This assay has also been criticised because the oxidative deterioration and antioxidant protection of fluorescent probe/target does not mimic a critical biological substrate (Frankel, 2007; Frankel and Meyer, 2000).

1.4.1.2 ET−based assays

ET-based assays involve two components in the reaction mixture, antioxidant and the oxidant (or probe). The reaction is based on the following electron-transfer reaction (Huang *et al.*, 2005):

probe (oxidant) + e- (from antioxidant) → reduced probe + oxidized antioxidant

The reaction probe itself serves as oxidant that abstracts an electron from the antioxidant, causing colour change of the probe. The degree of the colour change is proportional to the concentration as well as antioxidant potency of the antioxidant.

ET assays include Trolox equivalent antioxidant capacity (TEAC) assay, ferric reducing/antioxidant power (FRAP) assay, total phenols assay, ABTS radical scavenging assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and CUPRAC (cupric ion reducing antioxidant capacity) assay (Huang *et al.*, 2005; Prior *et al.*, 2005).

Total phenols assay by Folin-Ciocalteu reagent: The principle of total phenols assay involves electron-transfer reaction between phenolic compounds (or other reductants) and

molybdenum under alkaline conditions, resulting in the formation of blue complexes that can be detected spectrophotometrically at 725–765 nm (Magalhaes *et al*., 2008). Instead of measuring the total amount of phenolic compounds, the FCR actually reflect a sample's reducing capacity. In many plant and seaweed samples, a fairly good linear correlation was generally found between TPC and antioxidant activity evaluated by ET-based assays (such as DPPH, FRAP and TEAC). Accordingly, in our study, very high association was observed between the TPC of a variety of seaweed extracts and DPPH scavenging capacity ($R > 0.9$). There was also a strong correlation between TPC method and ORAC, a HAT-based assay (Wang *et al*., II). Therefore, the FCR assay was recently proposed for the determination of total reducing capacity of samples, which reflects the cumulative capacity of both phenolic and non-phenolic compounds to reduce the FC reagent (Prior *et al*., 2005; Magalhães *et al*., 2009).

FCR method has several advantages: it is easy to perform, inexpensive and reproducible. It has been reported that under proper conditions, this method produces a constant amount of blue pigment which is predictable from the reaction with suitable known phenols (Singleton and Rossi, 1965; Chun and Kim, 2004).

FCR assay is performed in aqueous media, thus it is not applicable for lipophilic antioxidants/matrices. It should also be noted that the Folin–Ciocalteau method is not an absolute measurement of the amount of phenolic compounds. A range of non-phenolic substances can interfere with this assay, resulting in overestimation of TPC compared to the chromatographic method (Prior *et al*., 2005). In addition, various phenolic compounds have different responses in this assay, which were affected by both the position and degree of hydroxylation (Ma and Cheung, 2007). The estimated TPC of the sample is also strongly dependent on the employed standard. If the standard used for calibration is highly reactive and gives a higher absorbance value, the calculated TPC values of the samples will be lower. Therefore, the choice of a suitable phenolic standard is important. With regard to the quantification of algal polyphenols (phlorotannins) in brown algae, the recommended standard is phloroglucinol monomer since phlorotannins are the only phenolic group in brown algae and phloroglucinol is the only commercially available standard. However, the use of phloroglucinol has been criticised because phlorotannins in seaweed samples consist of structurally distinct oligomers and polymers. When a polymeric mixture is analysed using a monomer as a standard, the response of shorter and longer polymers to FC reagent cannot be discriminated. The use of "self-standards" purified from the algal material itself has therefore been proposed (Appel, *et al*., 2001; Koivikko, 2008).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay: It was originally believed that the DPPH assay was a hydrogen transfer reaction but later studies suggested the reaction mechanism is mainly based on an ET reaction whilst the hydrogen atom abstraction is only a marginal reaction path (Foti *et al*., 2004; Huang *et al*., 2005).

The antiradical capacity of antioxidants in DPPH assay have been interpreted in many different ways including percent inhibition, percentage of residual DPPH, EC_{50} value, antiradical efficiency (AE) and ascorbic acid equivalent antioxidant capacity (AEAC). Although percent inhibition has still been widely used in some studies, direct comparison of the relative activity of different samples is not feasible. Another parameter commonly used is the EC_{50} value. EC_{50} value is independent of the sample concentration and thus is more useful than percent inhibition in comparing relative activity of samples with varying degrees of antioxidant activities. However, the linear relationship between percentage of inhibition and antioxidant concentration is valid only in very limited concentration range and special care is thus needed to accurately calculate the EC_{50} value. Another parameter used to characterise the antioxidant compounds is antiradical efficiency (AE, $AE = 1/(EC_{50}\, T_{EC50})$). It combines both stoichiometry (EC_{50}) and the time needed to reach the steady state to EC_{50} concentration (T_{EC50}) and has been shown to be a more discriminatory calculation of antioxidant scavenging capacity than EC_{50} (Sánchez-Moreno *et al*., 1998).

DPPH method has been used extensively as a simple and rapid method in pre-screening of new antioxidants from natural resources. The 2,2-diphenyl-1-picrylhydrazyl radical is stable, commercially available, and does not have to be generated before assay. Unlike other free radicals such as hydroxyl and superoxide anion radicals, the use of the stable DPPH radical has the advantage of being unaffected by certain side reactions, such as enzyme inhibition and metal ion chelation (Wettasinghe and Shahidi, 2000).

However, DPPH radicals are artificially generated, long-lived nitrogen radicals which bear no similarity to the highly reactive and transient peroxyl radicals. The ability of an antioxidant to scavenge DPPH radical may not reflect its real activity with food lipid radicals (Huang *et al*., 2005; Frankel, 2007). The reaction of DPPH with an antioxidant is strongly influenced by both the solvent and the pH of the reaction medium. Moreover, carotenoids presented in many plant extracts can interfere with the spectrophotometric measurements (Wettasinghe and Shahidi, 2000).

Reducing power assay: The reducing power of a compound has been found to be a significant indicator of its potential antioxidant capacity. A direct correlation between

antioxidant activities and the reducing power of certain plant extracts has been reported by several authors (Duh, 1998; Kumaran and Karunakaran, 2006).

Reducing power assay is simple to perform, inexpensive, speedy and reproducible. It can be easily automated and adapted to microplates, which allows analysis of large numbers of samples in one run.

Reducing power assay actually reflects only the ability of an antioxidant to reduce Fe (III) to Fe (II) in a redox linked colorimetric reaction, which is not physiologically and mechanistically relevant to the real activity of antioxidants in foods or biological systems. Although reducing power of a compound is an important feature for its overall antioxidant activity, not all reductants that are able to reduce Fe^{3+} are antioxidants. Conversely, an antioxidant that are highly effective in reducing the prooxidants may not be able to efficiently reduce Fe(III) (Prior and Cao, 1999). This method cannot detect antioxidants that act by radical scavenging, particularly some biologically relevant antioxidants (e.g. glutathione, cysteine and other thiol-containing compounds).

1.4.1.3 Ferrous ion-chelating ability

Ferrozine is a commercially available sulfonated ferroin and can combine with ferrous iron to form a stable complex. The absorbance of the complex is directly proportional to the iron concentration of the sample. Ferrous ion-chelating assay is simple to perform, inexpensive and has acceptable reproducibility. It is robust and can be performed using automated, semi-automatic or manual methods.

The major limitation of this method is that it does not evaluate other important properties of chelating agents which are essential to understand a compound's ability to suppress iron catalysed ROS generation, such as iron (III) selectivity, availability of coordination sites, affinity towards iron ions, rigidity of ligand conformations and stoichiometry of complex formation (Moore and Yu, 2008). Because the formation of ferrous-ferrozine complex is a time-dependent reaction, consistent timing between sample addition, mixing and absorbance measurement is required for optimal repeatability. Moreover, pigments present in plant and seaweed extracts may interfere with this method. In this case, a control sample which contains the plant or seaweed extracts (without the addition of ferrozine) should be included in each measurement (Wang *et al*., II).

1.4.1.4 Limitation of chemical reaction-based antioxidant tests

In recent years, there is growing concern about the claimed antioxidant effects of some natural antioxidants which are only based on simple and fast antioxidant tests. Many of these widely

used *in vitro* assays, for example, DPPH and ORAC, monitor scavenging activity against a single target radical under limited reaction conditions which are not relevant to real foods and biological systems and do not take into consideration important factors such as bioavailability, distribution, cellular uptake and metabolism of the antioxidants (Frankel, 2007; Frankel and Meyer, 2000). The reaction is generally carried out in the absence of any oxidation substrates such as lipids or proteins. These chemical antioxidant assays do not always predict the efficacy of the antioxidant in complex foods and biological tissues where multiple oxidative reactions may occur simultaneously and not only the intrinsic antioxidant property but also the physical location and orientation of antioxidant, the interaction with other food components and environmental conditions (e.g., temperature, pH and ionic strength) are crucial for the antioxidant effectiveness (Decker *et al*., 2005). These *in vitro* antioxidant tests are useful in initial screening of new antioxidants or in comparing different foods for their antioxidant capacity. But the antioxidant effectiveness of a potential antioxidant should not be based solely on the chemical antioxidant capacity and need to be verified in relevant model systems, eventually in real foods and biological systems.

1.4.2 Cell culture models to evaluate potential health effects

In vitro cell culture models have the advantage of relatively well-controlled variables and are generally more biologically relevant than the popular chemical reaction based assays because they reflect some aspects of cellular uptake, metabolism and location of antioxidant compounds within cells (Wolfe and Liu, 2007). Cell models have been demonstrated to be very useful for monitoring the quantity and through which cellular pathways the studied antioxidant was taken up by the cell. They also provide useful information about the mechanism of antioxidant reaction and dose-response relationships. Compared with full-scale clinical trials, cell-based assays are relatively cheap, fast and reproducible and therefore can serve as an intermediate testing methodology which has been increasingly used in recent antioxidant studies (Honzel et al., 2008).

Various cell-based systems have been developed to examine whether antioxidant candidates can limit ROS production or protect the cell from oxidative damage under physiologically relevant conditions. The cellular models frequently used for evaluation of antioxidant potential in foods and dietary supplements include human monocyte-based assay, cellular antioxidant activity (CAA) assay and cell-based antioxidant protection in an erythrocyte model (CAP-e) (Gunnarsson et al., 2006; Honzel et al., 2008; Wolfe and Liu, 2007). Among these cell-based systems, a cellular model comprising of human mononuclear cells and

isoluminol/luminol has been proven as a sensitive method for testing ROS scavenging activity of food-derived antioxidants (Gunnarsson et al., 2006). It is a high throughput method with sufficient reliability and reproducibility as biomarkers of *in vivo* oxidation (Raghavan et al., 2008).

However, cell behaviour is closely connected with source of cells, cell growth cycle and number of passes in cell culture. Complex interactions among different kinds of molecules in the cell may give rise to distinct cycles of activities. Molecules from other parts of the organism or even other organisms can also affect cell behaviour. The reproducibility of results within and across the laboratories can be poor (Schaich, 2008). Natural antioxidant mixture generally exhibit nonlinear dose-response in cell-based antioxidant assay. The overall antioxidant effect depends on the interaction between different antioxidant compounds in the tested samples and the type of the cell model. Simple numerical approaches commonly used in chemical antioxidant assays can not be used for the interpretation of complex, nonlinear dose-response relationship (Honzel et al., 2008).

To achieve a more consistent and reliable evaluation of the antioxidant performance of tested samples in complex cellular systems, the choice of appropriate cell culture model, the standardisation of the cell culture conditions and assay procedure as well as appropriate analysis and interpretation of nonlinear dose-responses data is of critical importance (Honzel et al., 2008).

1.4.3 Evaluation of antioxidant effects in food model systems

It is preferable that antioxidant study be performed under realistic conditions of food production, distribution and storage. However, the oxidative changes during storage are often too slow to be detected within a convenient time frame. Detailed studies of antioxidants in real food systems are complicated and usually difficult to control due to the complex nature of most food systems and could be influenced by many unknown factors (Frankel, 2005). It is not suitable for initial antioxidant screening experiments because it is expensive and time-consuming. Various simplified model systems such as bulk oils, emulsions, bilayer structures (microsomes, liposomes) and different types of skeletal muscle, which mimic the main features of a specific food system, have therefore been developed and widely employed to evaluate the antioxidant efficiency of potential antioxidants. In contrast to simplistic one-dimensional chemical assays, the antioxidant effectiveness of tested samples in model systems is evaluated in the presence of relevant oxidation substrates and therefore could provide much more useful information for predicting their performance in real foods or

biological systems. It must be stressed however that great care should be taken when selecting methods (markers) to monitor the oxidation process of the model and extrapolating the results obtained from model study to real food systems. The complexities of food and biological systems can often contradict the validity of the existing analytical methodology. To achieve a more comprehensive understanding of the precise antioxidant mechanisms, careful selection of model systems and monitoring of storage conditions is very important. It is also preferable to use more than one model system and more than one assay (Frankel, 2007).

1.4.3.1 Model systems for evaluating the antioxidant efficacy in muscle foods

Washed fish mince model system: Washed fish mince model has demonstrated to be an ideal matrix for oxidation study of fish muscle because it has intact myofibrillar proteins and membranes which resembles the structure of fish muscle, but has low content of triacylglycerols (Decker *et al*., 2005; Richards and Hultin, 2000; Undeland, 2003b). Sufficient washing with water and phosphate buffer can remove most aqueous pro- and antioxidants and the influence of endogenous pro- and antioxidants on the oxidation process is avoided. Exogenous oxidation catalysts (Hb) can then be added under controlled level to initiate lipid oxidation (Undeland, 2003a; Undeland, 2003b). Using washed fish model also allows easy manipulation of oxidation conditions such as pH, moisture content and temperature. The oxidative changes can be monitored within an appropriate experimental time frame. Compared with other model system such as bulk oil, emulsions or microsomes, the study of the antioxidant effectiveness of antioxidants in washed fish model can provide much more accurate predictions of their performance in real fish systems.

However, it has been reported that washed fish model occasionally fails to respond to Hb-mediated oxidation when they have very low moisture content (around 65-75%). This can bring some difficulties especially when large amount of tested antioxidant solution needs to be added into the model system. Results from a recently study showed that several noncontrollable and controllable factors are associated with this nonresponse, among which different α-tocopherol level and different degrees of disruption in the myofibrillar structure were suggested as the most important factors (Sannaveerappa *et al*., 2007). The washing step is not effective in removing endogenous membrane-bound antioxidant α-tocopherol, which located deep within the membrane. There are often interspecific and intraspecific variations in α-tocopherol contents of fish. The variations are correlated with seasons of catch, the size or the age of the fish, tissue type as well as the diet. Sannaveerappa *et al*. (2007) reported that α-tocopherol concentration of the nonresponding model was about two times higher than that of

the oxidising model. It was also observed that the nonresponding model had a more disrupted myofibrillar structure compared to its oxidising counterpart. Furthermore, they observed the absence of myocommata which suggested the onset of swelling in the stable model. The swelling or gelation could be a physical obstacle that prevents the added Hb to reach membranes. Therefore, special care should be taken during model preparation to control the degree of myofibrillar disruption, washing pH and washing temperature.

As discussed before, the mechanisms of lipid oxidation in fish muscle is very complex, involving not only Hb-catalysed systems but also oxidation initiated by other mechanisms. Hb-initiated lipid oxidation in washed fish muscle model does not reflect other mechanisms involved in the oxidation of real fish muscle, for example, the important role of LOXs and the cooperative actions of different prooxidative systems. An antioxidant that is highly effective in preventing Hb-initiated lipid oxidation may not be able to efficiently inhibit LOX activity.

2 OVERVIEW OF THE STUDIES

The thesis includes five studies as illustrated in Figure 2. Initially, an investigation was carried out to explore the effect of combined MAP and superchilled storage on the quality changes of fresh cod loins (I). Further, to enhance the quality of fish products with extended shelf life, the focus was shifted towards investigation on the potential of different types of underutilised Icelandic seaweeds as an optimal source of natural antioxidants for the development of new fish products. Extensive antioxidant studies were carried out on common Icelandic seaweeds to select the species with high antioxidant potentials (II). Extraction and separation techniques were evaluated and the antioxidant properties of algal polyphenols were characterised by *in vitro* antioxidant assays (II, III, IV). In the follow-up study, the antioxidant effectiveness of phlorotannin-containing extract and fractions was further tested in fish model systems (V). The possible health promoting effects were evaluated by measuring the ROS scavenging ability in a mononuclear cell-based bioassay (IV) and ACE inhibitory activity.

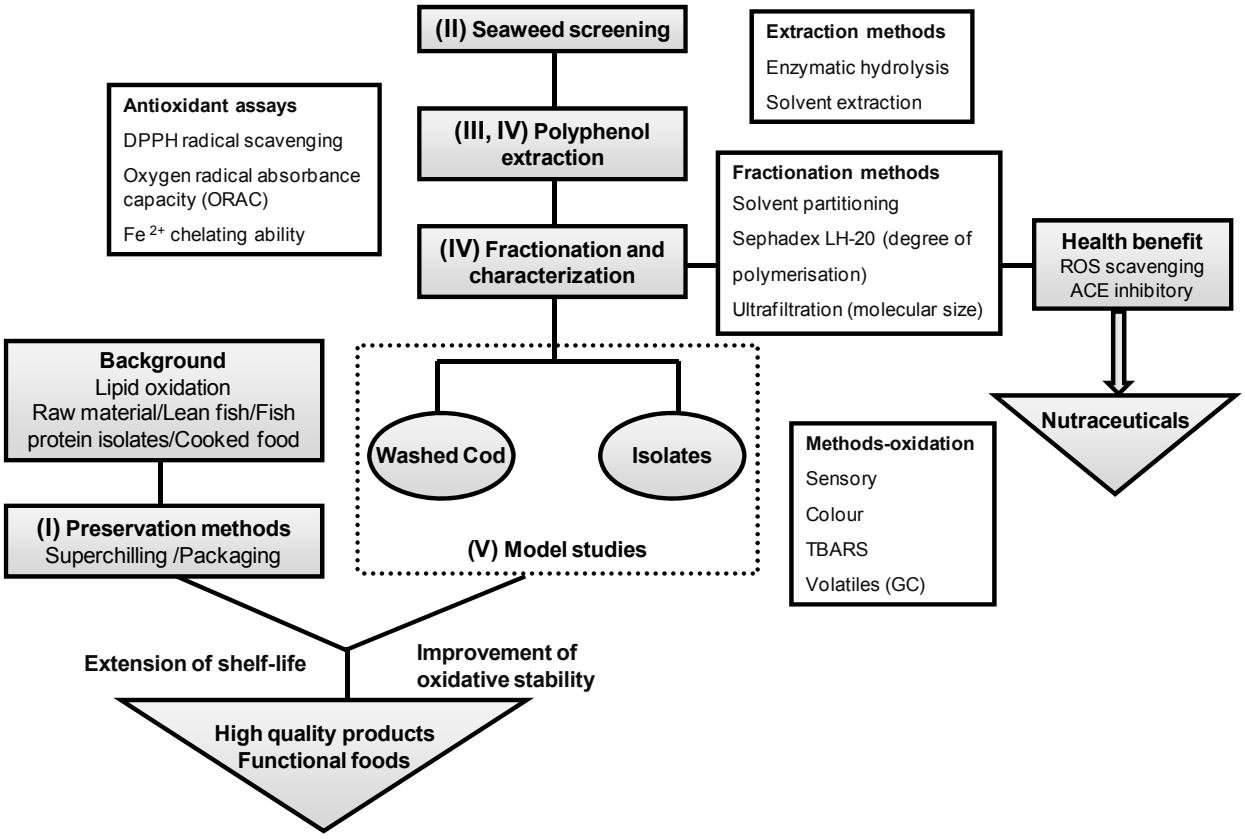


Figure 2. Overview of the study design

The innovation of the research was to characterise the antioxidant activity of algal polyphenols using a multiple-method approach which includes well documented *in vitro* antioxidant assays, cellular model as well as fish muscle and protein isolate model systems.

The aim was to get better understanding of antioxidant properties of polyphenols (especially phlorotannins) derived from marine algae and the relative contribution of algal polyphenols to the overall antioxidant activity of seaweed extracts. By using fish models which closely resemble the real fish systems, the results should allow better prediction of the antioxidant efficacy of seaweed extracts and partially purified fractions in real fish products.

The information gained is not only of academic interest but also of great practical value for the fish industry. The outcome of the study provides a necessary basis to enhance and broaden the potential utilisation of algal polyphenols in the development of novel fish products with improved oxidative stability, flavour quality and nutritional value as well as in health-promoting functional food formulations.

Paper I The objective was to study the shelf life and quality changes of fresh cod loins under MAP and superchilled storage conditions by sensory, physical, chemical and microbiological methods. The emphasis was on the evolution pattern of prime quality related sensory characteristics.

Paper II The antioxidant activities of ten species of common Icelandic seaweeds were evaluated by assaying for DPPH radical scavenging activity, ORAC and ferrous ion chelating ability. The correlations between TPC and antioxidant activities were studied in order to evaluate the role of algal polyphenol to the overall antioxidant activity and the possible mechanisms of antioxidant action.

Paper III The objective of the study was to investigate the effect of enzyme treatments on the extraction of polyphenols and other water-soluble antioxidant components from *P. palmata*. The potential to improve the antioxidant activity were assessed by three *in vitro* antioxidant assays.

Paper IV The efficiency of different solvent systems on the extraction of phlorotannins from *F. vesiculosus* was investigated. Further fractionation was carried out by solvent partitioning, Sephadex LH–20 chromatography and ultra-filtration. The antioxidant activity of different subfractions was characterised using a multiple-method approach, including *in vitro* antioxidant tests as well as a mononuclear cell-based bioassay.

Paper V The ability of various *F. vesiculosus* extract and fractions to inhibit hemoglobin-mediated lipid oxidation in washed cod muscle model and cod protein isolates was studied. The objective was to understand the relationships between the intrinsic antioxidant characteristics, molecular size and the antioxidant effectiveness in fish model systems.

3 MATERIALS & METHODS

3.1 Storage experiment

Fresh cod loins were obtained from Nyfiskur Company (Iceland). A total of 260 fresh cod loins were randomly divided into 4 sample groups and kept under different storage conditions. A1 and A2 were packed in polystyrene boxes; M1 and M2 were MA packed (50% CO_2 / 45% N_2 / 5% O_2). A1 and M1 were stored at 1.5 ± 0.2 °C; A2 and M2 were at -2.0 ± 0.3 °C from d 0 to d 4 and -0.9 ± 0.3 °C from d 4 to d 21. Packing and first sampling (d 0) was on day 3 post catch (I).

3.2 Sensory evaluation

The Quantitative Descriptive Analysis (QDA) method introduced by Stone and Sidel (1985) was used to evaluate the cooked samples of cod loins (I).

3.3 Chemical measurements

Total volatile basic nitrogen (TVB-N) and trimethylamine (TMA) were determined by the methods described by Malle & Poumeyrol (1989) (I).

3.4 Microbial analysis

Total viable psychrotrophic count (TVC) and H_2S-producing bacteria were enumerated on modified Long and Hammer's agar and iron agar (IA) as described by Van Spreekens (1974) and Gram *et al*. (1987). Nitrite–Actidione–Polymyxin (NAP) agar was used for counts of lactic acid bacteria (LAB) according to Davidson and Cronin (973) (I).

3.5 Algal materials

For antioxidant screening experiment, ten species of Icelandic seaweeds, including eight brown algae, two red algae and one green algae were collected in Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland on March 19th and May 16th, 2007 (Table 2, II). The red algae *Palmaria palmata* used for enzyme-assisted extraction study was collected from the same area on October 12th, 2007 (III) and the brown algae *Fucus vesiculosus* used

for solvent extraction, partially purification, characterisation and fish model study was collected on October 15th, 2008 (IV and V).

3.6 Solvent extraction

Based on our preliminary experiment and other studies, polar solvents were found to be more efficient to extract algal polyphenols than water and apolar solvents (Koivikko *et al*., 2005). Therefore, the extraction efficiency of various polar solvent systems was further compared in this study, including ethanol/water (80:20, v/v), ethanol/water (50:50, v/v), methanol/water (80:20, v/v), ethyl acetate/water (80:20, v/v) and acetone/water (70:30, v/v) (IV). The extraction was performed according to Wang et al. (II).

Table 2. List of seaweeds screened for antioxidant activity (II)

Scientific name	Common name	Icelandic name
Phaeophyta (Brown algae)		
1. *Fucus vesiculosus* Linnaeus	Bladder wrack	Bóluþang
2. *Fucus serratus* Linnaeus	Toothed wrack	Sagþang
3. *Ascophyllum nodosum* (Linnaeus) LeJolis	Knotted wrack; Egg wrack	Klóþang
4. *Alaria esculenta* (Linnaeus) Greville	Dabberlocks	Marinkjarni
5. *Laminaria hyperborea* (Gunnerus) Foslie	Cuvie; North European kelp	Stórþari
6. *Saccharina latissima* (Linnaeus) Lane, Mayes, Druehl and Saunders	Sugar kelp; Sea belt	Beltisþari
7. *Laminaria digitata* (Hudson) Lamouroux	Oarweed; Tangle kelp	Hrossaþari
Rhodophyta (Red algae)		
8. *Palmaria palmata* (Linnaeus) Kuntze	Dulse	Söl
9. *Chondrus crispus* Stackhouse	Irish moss	Fjörugrös
Chlorophyta (Green algae)		
10. *Ulva lactuca* Linnaeus	Sea lettuce	Maríusvunta

Fucus vesiculosus *Palmaria palmata* *Ulva lactuca*

(Picture: Karl Gunnarsson, with permission)

3.7 Solvent partitioning

The crude 80% ethanol extract was fractionated on the basis of polarity. The extract was partitioned successively with *n*-hexane, ethyl acetate (EtOAc) and *n*-butanol, yielding four fractions including the *n*-hexane-, EtOAc-, *n*-butanol-soluble fractions and the aqueous residue (Figure 3, IV and V).

3.8 Gel filtration on Sephadex LH–20 column

The EtOAc soluble fraction, which showed the highest TPC, DPPH scavenging and reducing capacity, was subjected to gel filtration on Sephadex LH–20 column and eluted stepwise with solvent systems of decreasing polarity from 50% aqueous MeOH, 75% aqueous MeOH, pure MeOH, MeOH/acetone 5:1, MeOH/acetone 3:1 to MeOH/acetone 1:1, yielding six subfractions (LH–1 to LH–6) (Figure 3, IV and V).

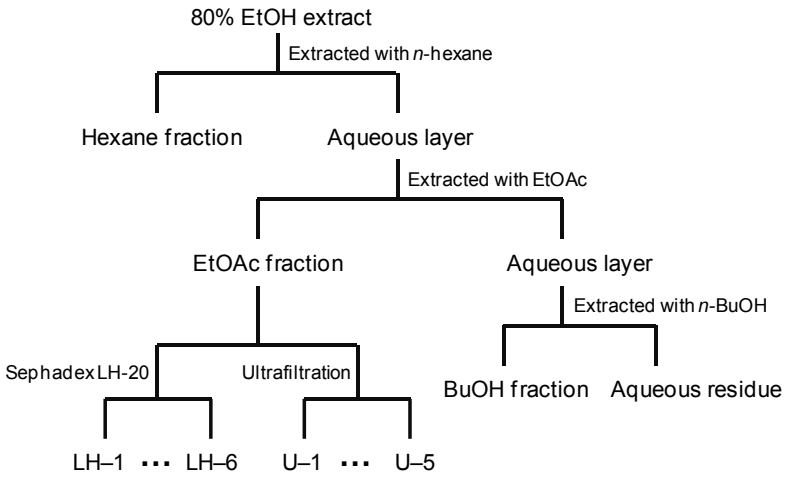


Figure 3. Flow diagram for extraction and fractionation of phlorotannins from *F. vesiculosus* (IV and V)

3.9 Preparation of different molecular weight subfractions

The EtOAc soluble fraction was also sub-fractionated into five fractions (>100, 30–100, 10–30, 5–10 and <5 kDa) of different molecular weight by using a series of Centricon filters (Figure 3, IV).

3.10 Enzyme-assisted extraction of algal polyphenols and other antioxidant components

3.10.1 Enzymatic extraction

Eleven commercial enzymes including six proteases and five carbohydrases were evaluated for extraction efficiency of hydrophilic antioxidant ingredients from the red algae *P. palmata* (III). The enzymatic extraction was performed according to the method of Heo *et al*. (2003) with slight modifications.

Eleven commercial enzymes as well as three carbohydrases produced by Prokaria, Matís ohf (Iceland) were compared for their efficiency in extracting phlorotannins from two brown algae, *F. vesiculosus* and *L. hyperborea*.

3.10.2 Fractionation of Umamizyme hydrolysate of *P. palmata*

Umamizyme hydrolysate was further fractionated into three subfractions, namely crude polyphenol, crude polysaccharide and LMW aqueous fractions and the antioxidant activity was evaluated in order to gain a basic understanding of the relative contribution of different constituents to the overall antioxidant capacity of *P. palmata* hydrolysate (III).

3.11 Determination of total phenolic content

The total phenolic content (TPC) of seaweed extracts and fractions was determined in accordance with a protocol described by Turkmen *et al*. (2005) and Koivikko *et al*. (2005) (II, III,). Many studies have shown that phlorotannins are the only phenolic group detected in brown algae (Jormalainen and Honkanen, 2004; Koivikko *et al.*, 2007). Folin-Ciocalteu colorimetric method has also been widely used to estimate the total phlorotannin content in brown algae. Therefore, TPC represented total phlorotannin content of *F. vesiculosus* extracts and fractions in IV and V.

3.12 *In vitro* antioxidant assays

3.12.1 DPPH radical scavenging activity

The method of Sánchez-Moreno *et al*. (1998) was used for measuring the DPPH radical scavenging ability of seaweed extracts and fractions. L-ascorbic acid, BHT and α-tocopherol were used as reference standards (II, III, IV and V).

The calibration curve made with DPPH• between 4.15×10^{-3} and 4.15×10^{-2} g/l was used to calculate the remaining concentration of DPPH• in the reaction medium.

The EC_{50} value was calculated as the concentration of sample or standard antioxidant (mg/ml or µg/ml) required to scavenge 50% of the DPPH• in the reaction mixture. Its reciprocal, the antiradical power (ARP, ARP = $1/EC_{50}$) was also calculated.

3.12.2 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Davalos *et al*. (2004) with slight modifications. Mx300 real-time PCR System (Stratagene Inc., La Jolla, CA) was used for the fluorescence measurements controlled by MxPro computer program (II, III).
The ORAC value was calculated and expressed as micromoles of Trolox equivalents per gram extract (µmol of TE/g extract) using the calibration curve of Trolox.

3.12.3 Ferrous ion-chelating ability assay

The Ferrous ion-chelating ability was determined according to the method of Decker and Welch (1990) with minor modifications. L-ascorbic acid, citric acid and EDTA-Na_2 were used as reference standards (II, III, IV and V).

3.12.4 Reducing power

The reducing power was measured as described by Benjakul *et al*. (2005). The results were expressed as mg of ascorbic acid equivalents (ASE) per gram of extract or fractions (IV and V).

3.13 Fish model study

3.13.1 Preparation of hemolysate and quantification of haemoglobin

Hemolysate was prepared within 24 h of blood collection according to the method of Richards and Hultin (2000). The concentration of haemoglobin was determined by the HemoCue system of plasma/low Hb microcuvettes and photometer (Jónsdóttir *et al*., 2007). A standard curve with serial bovine haemoglobin solution (ranging from 0-70 µmol/l) was used for calibration (V).

3.13.2 Washed cod mince

The washed cod mince was prepared according to the method of Richards and Hultin (2000) (V).

3.13.3 Preparation of protein isolates by alkaline solubilisation

Protein isolate model was prepared from cod white muscle according to Kristinsson *et al*. (2005) with some modifications (V).

3.13.4 Preparation of oxidation system

The preparation of oxidation system was based on Richards and Hultin (2000) and Undeland et al. (2004). Seaweed extract and fractions were added at 300 mg/kg model. Propyl gallate (PG) was added at 100 mg/kg model. 50% aqueous ethanol (v/v) was used as carrier solvent at 1% of the final model weight (V).

3.13.5 Thiobarbituric Reactive Substances (TBARS)

Samples were taken periodically according to the procedure described by Undeland *et al*. (2002). TBARS were determined according to direct extraction method (Sørensen and Jørgensen, 1996). TBARS values, expressed as μmol malondialdehyde per kg of sample (μmol MDA/kg), was calculated using malondialdehyd-bis-(diethyl acetate) as a standard (V).

3.13.6 Sensory analysis

Four to five trained panelists participated in the sensory assessments. Sensory evaluation was conducted under standardised conditions. Capped samples were allowed to rest for 45 min on ice between panelists' sessions, in order to recover equilibrium in the headspace of the samples. The panelists judged the samples according to the intensity of rancid odour on an unscaled line (100 mm long) ranging from not detectable (0) to dominant (100) (V).

3.13.7 Volatile compounds by gas chromatography

Sample was weighed (4.5 – 5.0 g) into a 5 ml sealed vial for headspace Solid Phase Microextraction (HS-SPME) / Gas Chromatography analysis (GC-O and GC-FID). Heptanoic acid ethyl ester was used as an internal standard. Separation, identification and quantification of volatile compounds collected by the SPME technique was done by combined GC-O and GC-FID analysis as described by Jónsdóttir *et al*. (2007) (V). Gas Chromatography-Mass Spectrometry (GC-MS) was used to confirm the identity of volatiles but not for quantification.

3.14 Chemiluminescence assay for the detection of reactive oxygen species

Mononuclear cells were isolated from the blood of human volunteers using BD Vacutainer method as described by Raghavan *et al*. (2008). Chemiluminescence (CL) technique (Gunnarsson et al., 2006) was used for detecting ROS produced by PMA-stimulated human mononuclear cells.

3.15 ACE inhibitory activity

ACE inhibitory activity was measured according to Vermeirssen *et al*. (2002) with modifications. In brief, 20 µl sample or standard solution was mixed with 10 µl of 0.2 U/ml angiotensin converting enzyme from rabbit lung (Sigma-Aldrich, St. Louis, MO) and the mixture solution was pre-incubated at 37°C for 15 min in a microplate. Subsequently 170 µl of the substrate solution (0.5 mM N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly in 50 mM Tris-HCl buffer, pH 7.5, containing 300 mM NaCl) was added with a multi-channel pipette. The microplate was quickly placed in a microplate reader (POLARstar OPTIMA, BMG LABTECH GmbH, Offenburg, Germany) and the absorbance at 340 nm was recorded every minute for 60 min. The microplate reader was thermostated at 37 °C and the plate was automatically shaken prior to each reading. 20 µl Tris-HCl buffer (50 mM, pH 7.5, with 300 mM NaCl) instead of sample solution was used as a control. The ACE inhibitory activity (%) was calculated as

$$(1-(\Delta_{sample} / \Delta_{control}) \times 100)$$

Where Δ_{sample} is the slope of the sample with inhibitor and $\Delta_{control}$ is the slope of the control. The concentration of extract/fractions needed to inhibit the ACE by 50% (IC_{50}) was determined by assaying algal samples at different concentrations and plotting the ACE inhibition percentage as a function of sample concentration, as described by Shalaby *et al*. (2006).

3.16 Statistical analysis

Analysis of variance (ANOVA) was applied to the experimental data set using the Number Cruncher Statistical Software, NCSS 2000 (NCSS, Kaysville, Utah, USA). Significant differences were determined by one way ANOVA and Duncan's Multiple-Comparison Test or Tukey-Kramer Multiple-Comparison Test was used to determine the statistical difference

between sample groups. Significance of differences was defined at the 5% level ($p<0.05$) (I, II, III, IV and V).

Sensory attributes were analysed using Principal Component Analysis (PCA) in the statistical program Unscrambler® (Version 9.5, CAMO, Trondheim, Norway). Full cross-validation was used in the validation models (I).

Multivariate comparison of TPC and antioxidant activity of 10 species of algae was performed by the Unscrambler 9.7 software package (CAMO AS, Trondheim, Norway). The main variance in the data set was detected using PCA. All data were mean centred and scaled to equal unit variance prior to PCA. Full cross-validation was used in the validation models (II).

Partial least square regression (PLSR) was used to test the quantitative correlation between TPC and antioxidant properties of all the water and 70% acetone extracts. DPPH, ORAC and chelating activity were used as *X* predictors and TPC as *Y* response factor (II).

The panelists' performance was analysed using PanelCheck V1.3.2 (V).

4 RESULTS & DISCUSSION

4.1 Combined application of modified atmosphere packaging (MAP) and superchilled storage on the quality of fresh cod loins

4.1.1 Shelf-life extension

An overview of the initial values for the microbial, TVB-N, TMA, pH and estimated values at sensory rejection for all experimental groups as well as shelf-life estimation are shown in Table 3 (I). Based on the sensory evaluation of cooked cod loins by QDA (average QDA scores for attributes related to spoilage = 20), the expected total shelf-life (days from catch) of traditional chill-stored cod loins was about 9 d. Chilled MA packaging and superchilled storage alone increased the shelf-life to 14 d and 16 or 17 d, respectively. When MAP and superchilled storage were combined, a synergistic effect was observed and the total shelf-life was further extended to at least 24 d. When using TVB-N value (35 mg N/100g) as a criterion to determine the end of shelf-life, a relatively shorter shelf-life was estimated in agreement with earlier studies (Olafsdóttir *et al*., 2006). The difference between sensory and chemical estimated shelf life is around 1 d for chill and superchill-stored samples and 2 d for chilled MA packaging group (Table 3, I).

Table 3. Overview of shelf-life estimation, measured initial values for the microbial, TVB-N, TMA, pH and final estimated values at sensory rejection for all experimental groups. (I)

	Initial values	Estimated values at sensory rejection			
		A1	A2	M1	M2
Estimated shelf-life (d)[a]		9	16-17	14	>24
Estimated shelf-life(d)[b]		8	16	12	24
TVC (log_{10}CFU/g)	4.7	7.5	7.9-7.9	7.4	>7.4
H_2S-producer counts	2.6	6.7	7.5-7.5	5.7	>5.5
LAB counts	Nd	5.6	6.4-6.5	6.2	>6.0
TVB-N (mg N / 100g)	10.8	42.0	36.6-59.2	46.1	>36.3
TMA (mg N / 100g)	0.0	30.2	25.5-44.8	33.4	>24.0
pH	6.63	7.15	6.99-7.14	6.97	>6.84

a) Total shelf life, including days from catch, based on the sensory evaluation of cooked loins (average QDA scores for attributes related to spoilage = 20)
b) Total shelf life, including days from catch, based on TVB-N = 35 mgN/100g

4.1.2 Retaining the prime quality characteristics

PCA analysis was carried out to study the sensory characteristics of different sample groups. The samples differed mainly with regard to odour and flavour attributes along PC1 which appeared to be substantially linked to the duration of storage. At the beginning of storage, the sweet odour and flavour, metallic flavour, boiled milk and vanilla odour were prominent. These characteristics became less evident as storage time progressed, but least change was observed in superchilled MA packed samples (M2) as seen by location of all M2 samples on the left side of the plot before d 15 (Figure 4, I). PC2 primarily explained variation between samples with regard to texture attributes. Superchilled MA packed cod loins had more meaty texture compared to other sample groups.

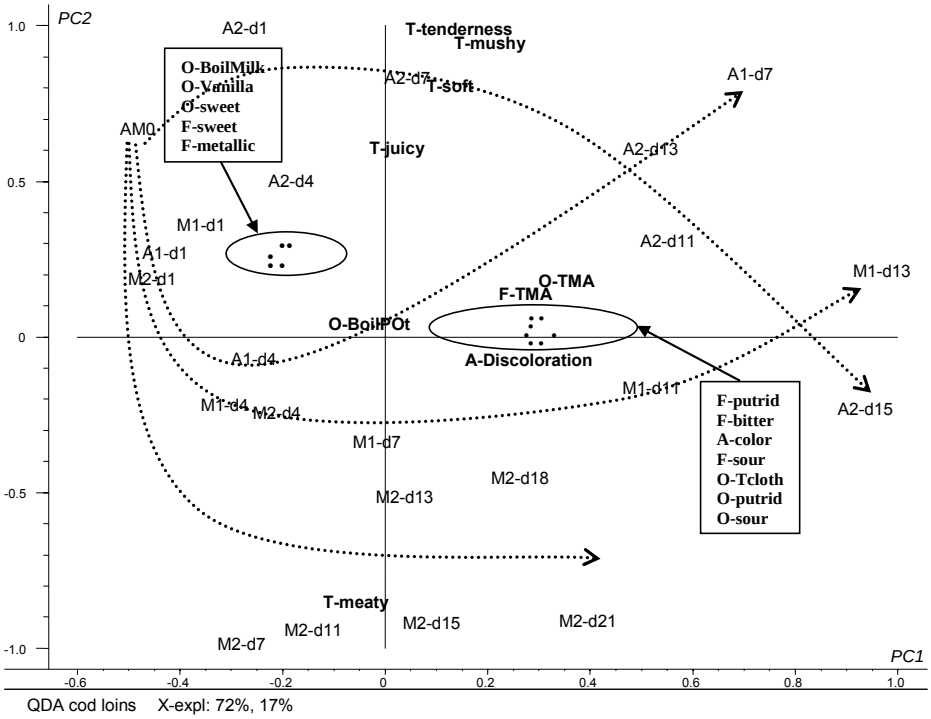


Figure 4. PCA: Bi-plot of scores (samples) and loadings (sensory attributes). AM0-initial sample; F = Flavour, O = Odour, A = Appearance, T = Texture; Dotted lines indicate evolution of each sample group with storage time. (I)

It was also noted that the superchilled MA packed sample (M2) received significantly higher scores for sweet, boiled milk and vanilla odour, sweet and metallic flavour on days 11, 13 and 15 ($p<0.05$) than groups A2 and M1 (I). This is also clearly shown in Figure 5 and Figure 6. The combination of MAP with superchilled storage resulted in a better maintenance of characteristic "fresh fish" odour and flavour after 11 d storage, compared with MAP or superchilled storage alone. This is of great important because MA packaging usually extends

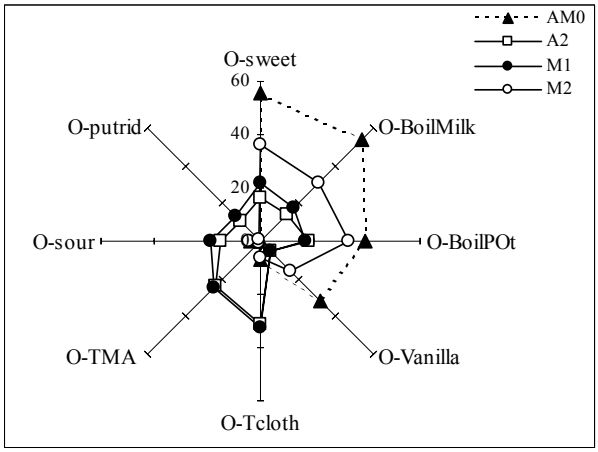


Figure 5. Radar plot of QDA odour attributes (mean scores) of different storage groups on d 11. AM0-initial sample; O=Odour; Groups M1 was stored at 1.5 °C; A2 and M2 at -1.0 °C. (I)

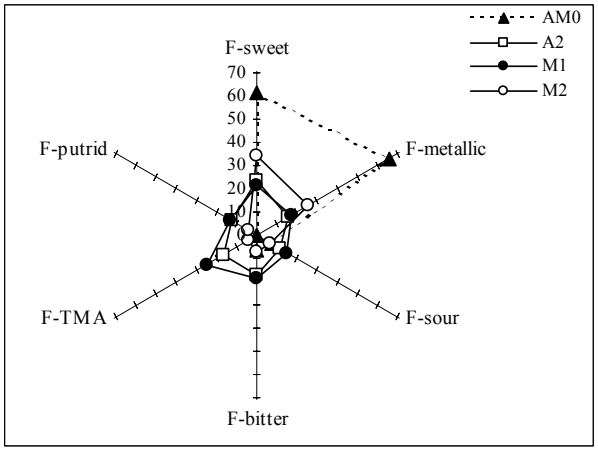


Figure 6. Radar plot of QDA flavour attributes (mean scores) of different storage groups on d 11. AM0-initial sample; F=Flavour; Groups M1 was stored at 1.5 °C; A2 and M2 at -1.0 °C. (I)

the period of moderate to low quality (neutral taste period) rather than earlier stage of prime quality (Sivertsvik *et al*., 2003).

4.1.3 Limitations and future research

In this study, MAP combined with superchilled storage led to the change of the textural properties. Superchilled MA packed cod loins had more meaty texture compared to other sample groups after 7 d storage, which could be explained by the temperature fluctuation during the first 4 d storage. Strict temperature control is crucial to avoid negative impact on the texture caused by ice crystal formation when the storage temperature is approaching the freezing point of the fish (I).

Most of the previous studies on the new preservation technologies have mainly focused on sensory, microbiology and chemical analysis. Few studies have been carried out on lipid oxidation and enzymatic degradation under MA or superchilled storage. The application of MA packaging, superchilling and storage at subzero temperatures can effectively control microbial growth and extend the shelf life of fish and fish products. However, the spoilage pattern may also change. Oxidative changes and enzymatic degradation may not be effectively inhibited or even become more pronounced, limiting the shelf life. Increased enzymatic activity has been reported to occur in superchilled fish (Foegeding *et al*., 1996). A better understanding of the process of quality deterioration and the methods to delay and/or prevent lipid oxidation and enzymatic degradation is a potentially interesting topic for future research.

4.2 Screening of antioxidant activity in Icelandic seaweeds

To justify the selection of the most appropriate species with high antioxidant potential for possible application as natural antioxidants in fish products or marine functional food ingredients, the pre-screening experiment was carried out using ten species of common seaweeds found on Icelandic seashores.

Significant differences in total phenolic content (TPC) were observed among different seaweed species. Brown algae generally contained higher amounts of polyphenols than red and green algae. Fucoid species, especially *F. vesiculosus* and *F. serratus* were found to be richest in polyphenols (II).

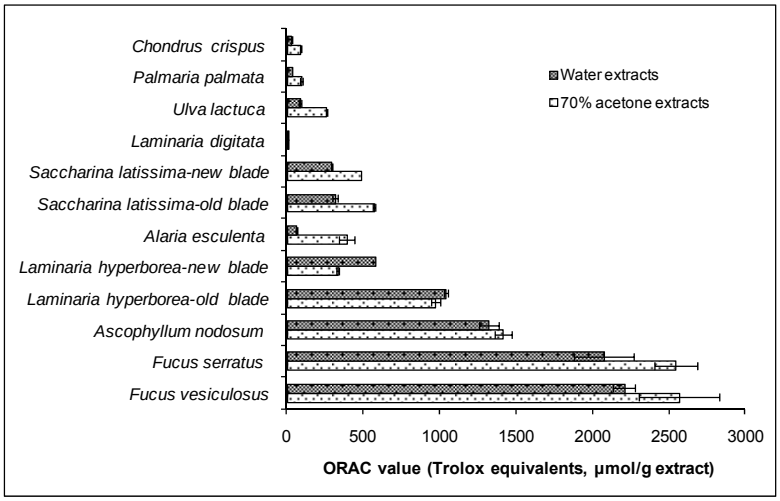


Figure 7. Oxygen radical absorbance capacity (ORAC) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds. Values are means ± S.D. (n = 3). (II)

The radical scavenging activities evaluated by both DPPH and ORAC showed similar trends (II). Among the seaweeds studied, 70% acetone extract of *F. vesiculosus* and *F. serratus* exhibited the highest scavenging activity against DPPH and peroxyl radicals (Figure 7, II). Because of lack of ORAC data on seaweeds, our results cannot be evaluated by comparing with ORAC values of other seaweed species. However, the ORAC values of the fucoid species in the present study were comparable to or even higher than those reported in the literature for various high antioxidant fruit and spice extracts (Huang *et al.*, 2002; Elisia *et al.*, 2007; Su *et al.*, 2007).

The ferrous ion-chelating activity of seaweed extracts showed a very different pattern when compared with DPPH and ORAC results. All the water extracts were more effective in chelating ferrous ion than their acetone extract counterparts (Figure 8, II). It was also noticed that water extracts from two red algae (*P. palmata* and *C. crispus*) with significantly lower

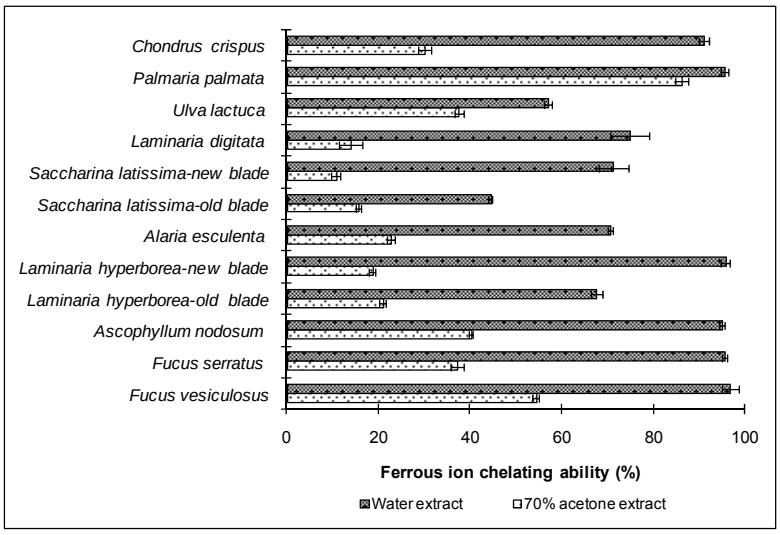


Figure 8. Ferrous ion-chelating activity of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds. Values are means ± S.D. (n = 3). (II)

levels of TPC as well as weaker radical scavenging activities, exhibited equivalent chelating effects as fucoid species.

PCA was carried out to gain a clearer overview of the similarities and differences among different algal species and to investigate the correlation between TPC and different antioxidant activities (II). Combining the information obtained from the score and the loading plots, it can be concluded that different seaweed samples can be discriminated and ranked according to their TPC, ORAC and DPPH along PC1 (Figure 9, II). It was noted that TPC, ORAC and DPPH radical scavenging activity were closely loaded on PC1, indicating that TPC and free radical quenching activities were highly correlated with each other (II). PC2 mainly explained variation between samples with regard to ferrous ion-chelating ability. Moreover, ferrous ion-chelating capacity loaded heavily on the second component while TPC had low loading, which illustrated that no clear correlation existed between TPC and chelating capacity.

Pearson correlation analysis further demonstrated strong correlations between TPC, DPPH radical scavenging activity and ORAC value of seaweed extracts. On the other hand, ferrous ion-chelating ability of seaweed extracts correlated neither with TPC, nor with DPPH or ORAC (II).

Based on these analyses, it can be speculated that algal polyphenols mainly act as potent free radical scavengers and primary, chain-breaking antioxidants. In contrast to what has been reported by Chew et al. (2008), our results showed that algal polyphenols are probably not effective metal chelators as other algal components such as high molecular weight polysaccharides, proteins or peptides (II).

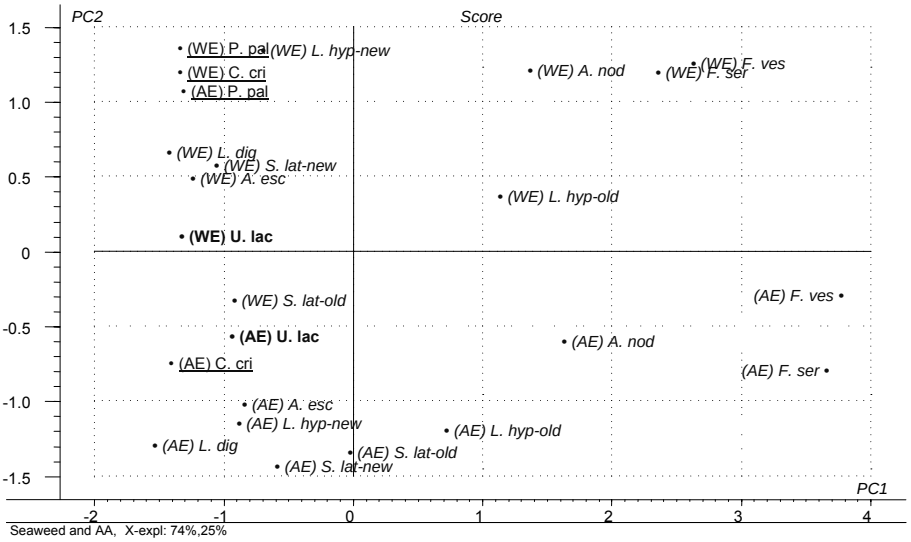


Figure 9. PCA score plot of total phenolic content (TPC) and antioxidant activities (AA) (DPPH, ORAC and ferrous ion-chelating activity) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds (F. ves, *F. vesiculosus*; F. ser, *F. serratus*; A. nod, *A. nodosum*; L. hyp, *L. hyperborea*; A. esc, *A. esculenta*; S. lat, *S. latissima*; L. dig, *L. digitata*; U. lac, *U. lactuca*; P. pal, *P. palmata*; C. cri, *C. crispus*). Brown seaweeds are indicated by italics; red seaweeds by underline; green seaweeds in bold font. (II)

4.3 Extraction of algal polyphenols

This section reports the preliminary results on factors influencing TPC of seaweeds and a more detailed study of the effect of solvent type on the extraction efficiency of algal polyphenols.

Significant intraplant variations in TPC of brown algae are well documented. The variations are species-dependent and influenced by plant size, age, tissue type as well as environmental factors such as salinity, nutrient and light availability (Pavia and Toth, 2000). In the screening experiment, intra-thallus variations in TPC were observed both in *L. hyperborea* and *S. latissima*. Old blades had higher TPC values compared to new blades (II). This is in agreement with earlier studies by Connan *et al*. (2004) who reported that there was a high correlation between the age of the tissues and TPC for *L. hyperborea*. Large seasonal fluctuations in TPC have been reported for brown fucoid algae (Pavia and Toth, 2000). The TPC is generally higher in summer and early autumn whereas lower levels were observed during winter, supporting the important photo-protective roles of phlorotannins. In the present study, we observed that TPC levels of both water and 70% acetone extracts from *F. vesiculosus* samples collected in October, 2008 were much higher than those collected in

early spring (March, 2007) (II and IV). The information is of practical importance for future utilisation and production of seaweed-based antioxidants and warrant furthered investigation.

The type of extractant had an impact on the amount of total extractable substances (TES). For most screened seaweed species, the TES of water extracts were higher than those of 70% acetone extracts and large differences were observed for *U. lactuca*, *P. palmata* and *C. crispus* (II). These seaweeds are known to contain high levels of water-soluble components, such as soluble polysaccharides, protein and peptides (Galland-Irmouli *et al*., 1999) which were poorly extracted by 70% acetone. With regard to *F. vesiculosus*, TES generally decreased with decreasing polarity of the extractant (IV).

Type of solvent also influenced the extraction efficiency and selectivity of phenolic compounds. But no direct relationship was observed between the amount of TES and the TPC of the extracts. For most seaweed species, TPC of 70% acetone extracts was higher than those of water extracts (II). Polar solvents were more efficient to extract phlorotannin compounds from *F. vesiculosus* than water and apolar solvents and the highest TPC was recorded in 70% acetone extract (IV and unpublished data). However, 80% ethanol also showed considerable ability to extract phlorotannins and the use of ethanol would obviously be preferred for the extraction of food-grade natural antioxidants.

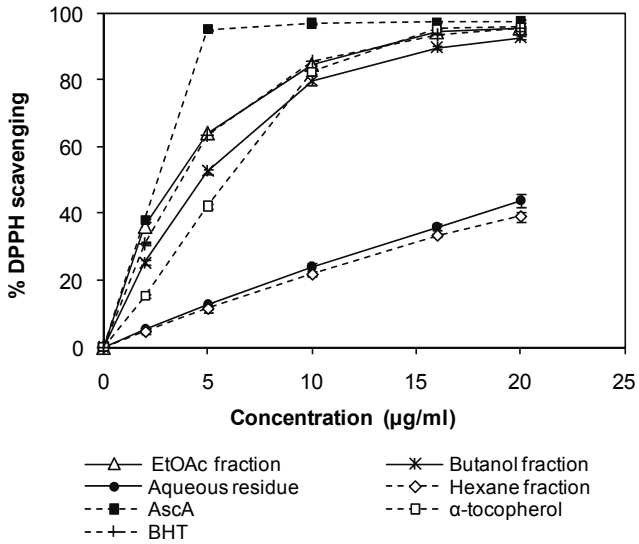


Figure 10. DPPH radical scavenging activity of different solvent fractions of *F. vesiculosus* compared with standard antioxidants. Values are means ± S.D. (n = 3). (IV)

4.4 *In vitro* antioxidant activity–different solvent fractions

Solvent partitioning was carried out to evaluate the role of phlorotannins in the antioxidant activity of the crude 80% ethanol extract from *F. vesiculosus* and to test the effectiveness of EtOAc in concentrating phlorotannins. Among the four fractions obtained, the EtOAc-soluble fraction showed the highest TPC, followed by the *n*-butanol fraction and aqueous residue. The lowest level of TPC was found in the *n*-hexane fraction (IV). Ethyl acetate has been widely used to selectively extract polyphenolic compounds of intermediate polarity from various plants. The results of the present study also demonstrated the effectiveness of EtOAc in concentrating/enriching phlorotannins from crude *F. vesiculosus* extract. In accordance with TPC result, the highest DPPH scavenging and reducing activities were detected in EtOAc fraction, whilst the other fractions showed lower activities (Figure 10, IV).

4.5 *In vitro* antioxidant activity–phlorotannin oligomers versus polymers

Phlorotannins from *F. vesiculosus* were observed to have a strong adsorption affinity for the Sephadex LH-20 gel, mainly due to the formation of hydrogen-bonding between the phenolic hydroxyl groups and the ether oxygen atoms of the gel (Kantz *et al*., 1990; Shahidi and Naczk, 2004b**)**. Phlorotannin polymers containing many phenolic hydroxyl groups are adsorbed more strongly on the column than monomers and oligomers. Using appropriate eluent systems allows separation of oligomers and polymers based on hydrogen-bonding properties. In this study, phlorotannin oligomers were eluted first from the gel by aqueous methanol. Gradual desorption of polymeric phenols was achieved by stepwise increase in the acetone concentration of acetone–methanol elution mixture. The carbonyl oxygen of acetone has been reported to serve as a strong hydrogen bond acceptor and enables displacement of complexed polymeric phenols from Sephadex LH-20 column (Jerez *et al*., 2007).

The majority of the phlorotannin constituents in EtOAc fraction were eluted from the column in the last three subfractions, accounting for about 78% of the total amount recovered (Table 4, IV). It may therefore be assumed that phlorotannins in *F. vesiculosus* comprise mainly polymeric phlorotannins.

Different Sephadex subfractions showed varying degrees of antioxidant activities (Table 4, IV). The highest DPPH radical scavenging activity was found in LH–2 and LH–3, which was comparable to those of the positive controls AscA and BHT, and was significantly higher than that of α-tocopherol. There was no clear trend in the chemical antioxidant activity among

Table 4. Relative yields, total phlorotannin content (TPC) and antioxidant activities of different Sephadex subfractions from EtOAc fraction of *F. vesiculosus*. (IV)

Subfractions	Relative yields (%) [2)]	TPC (g PGE/100 g) [3)]	DPPH EC_{50} (µg/ml) [4)]	Fe^{2+} chelating ability (%)	Reducing power (mg ASE/g) [5)]
LH–1	7.3 ± 1.3^{d}	82.7 ± 1.5^{d}	4.23 ± 0.17^{a}	46.5 ± 1.4^{a}	760.3 ± 63.9^{a}
LH–2	7.5 ± 0.5^{d}	96.6 ± 0.9^{a}	2.79 ± 0.05^{d}	28.5 ± 0.4^{e}	790.3 ± 61.3^{a}
LH–3	7.4 ± 0.9^{d}	97.0 ± 0.7^{a}	2.82 ± 0.03^{d}	30.5 ± 0.8^{de}	822.3 ± 29.2^{a}
LH–4	12.7 ± 0.3^{c}	85.0 ± 0.9^{cd}	3.89 ± 0.07^{b}	32.3 ± 1.1^{d}	717.8 ± 56.4^{a}
LH–5	46.2 ± 2.4^{a}	91.3 ± 2.4^{b}	3.50 ± 0.13^{c}	38.0 ± 0.5^{c}	785.8 ± 77.1^{a}
LH–6	18.9 ± 2.0^{b}	87.5 ± 1.2^{bc}	3.71 ± 0.07^{bc}	41.9 ± 1.8^{b}	780.5 ± 82.1^{a}
Total % recovery [1)]	75.4 ± 1.3				

Each value is expressed as means ± S.D. (n = 3).
Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).
[1)] Total % recovery from 300 mg of EtOAc fraction loaded on the Sephadex LH-20 column.
[2)] Relative yields are expressed as (%) weight of individual freeze-dried fraction.
[3)] TPC, g phloroglucinol equivalents (PGE) /100g extract.
[4)] EC_{50}, concentration of extract (µg/ml) required to scavenge 50% of the DPPH• in the reaction mixture.
[5)] ASE, ascorbic acid equivalents.

oligomeric and polymeric phlorotannin-rich subfractions, but the differences in the reducing and DPPH scavenging activities appeared to be explained by the relative TPC levels. Phlorotannin oligomers and polymers did not seem to differ markedly in chemical antioxidant activity. This is in agreement with a previous study that found no association between the type of structural linkages and DPPH scavenging activity of phlorotannins isolated from another Fucoid species, *F. spiralis* (Cerantola *et al*., 2006).

4.6 Is there any correlation between MW and *in vitro* antioxidant activity?

At present, very little information is available concerning the MW distribution of phlorotannins in *F. vesiculosus* as well as the relationship between MW and antioxidant activity. In this study, EtOAc-soluble fraction was fractionated by sequential ultrafiltration through Centricon membranes of different NMWL to yield five subfractions. Subfraction with MW > 100 kDa was the most abundant and represented about 73.9% of the total amount recovered, indicating that highly polymerised phlorotannins are the largest pool of phenolic compounds in *F. vesiculosus* (Table 5, IV).

Subfractions with high levels of TPC such as 10-30 kDa, 30-100 kDa and 5-10 kDa, generally exhibited strong DPPH radical scavenging activity and reducing power (Table 5, IV). There

Table 5. Relative yields, total phlorotannin content (TPC) and antioxidant activities of different MW subfractions from EtOAc fraction of *F. vesiculosus*. (IV)

Subfractions	Relative yields (%) [a]	TPC (g PGE/100 g)	DPPH EC_{50} (μg/ml)	Fe^{2+} chelating ability (%)	Reducing power (mg ASE/g)
> 100 kDa	73.9 ± 1.6[a]	87.8 ± 2.3[b]	3.80 ± 0.13[a]	45.1 ± 1.0[a]	881.3 ± 40.2[a]
100-30 kDa	9.6 ± 0.6[b]	95.4 ± 1.3[a]	3.58 ± 0.07[a]	47.6 ± 1.3[a]	899.0 ± 38.0[a]
30-10 kDa	3.3 ± 0.3[c]	97.0 ± 1.5[a]	3.07 ± 0.04[b]	36.6 ± 1.8[b]	910.7 ± 27.5[a]
10-5 kDa	2.7 ± 0.2[c]	94.8 ± 0.5[a]	2.91 ± 0.09[b]	33.7 ± 0.6[b]	822.0 ± 40.3[ab]
< 5 kDa	10.4 ± 0.9[b]	83.1 ± 1.8[b]	3.62 ± 0.11[a]	25.1 ± 0.2[c]	734.0 ± 43.0[b]

Each value is expressed as means ± S.D. (n = 3).
Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).
[a] Relative yields are expressed as (%) weight of individual freeze-dried fraction

seemed to be no direct relationship between the molecular size of phlorotannin compounds and *in vitro* antioxidant activity.

4.7 Enzyme-enhanced extraction of antioxidant components from *P. palmata*

In this study, eleven commercial enzymes were evaluated for the extraction efficiency of hydrophilic antioxidant compounds from *P. palmata*. All the proteases tested showed greater capacities to enhance the recovery of phenolic compounds than water extraction. On the contrary, the TPC of the hydrolysates prepared with carbohydrases were generally lower. In particular, Umamizyme exhibited the highest extraction efficiency of polyphenols, which was approximately 3.0-fold higher than that of the water extract. The relatively lower TPC of both water and carbohydrase extracts could be partly due to the formation of protein–polyphenol complexes during the extraction (Siriwardhana *et al*., 2008). The use of proteases substantially converts proteins into small peptides and free amino acids and reduce the complex formation between protein and algal polyphenols, which could explain the improved extraction efficiency of phenolic compounds by proteases observed in the present study. Another explanation for the decreased TPC in carbohydrase extracts could be due to the release of oligosaccharides and simple sugars during the degradation of cell wall polysaccharides (Figure 11, III).

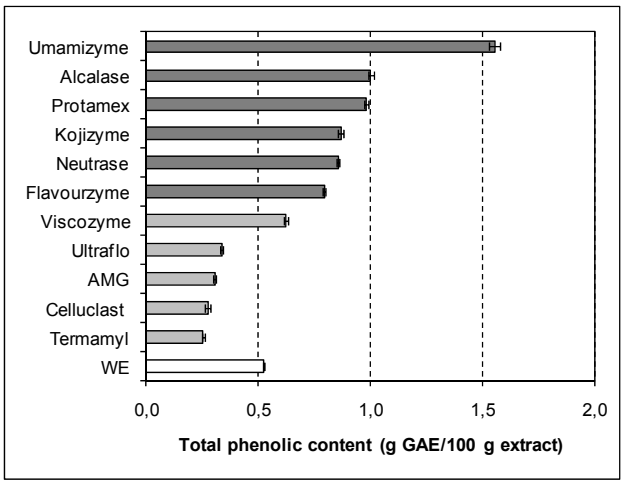


Figure 11. Total phenolic content (TPC) of different enzymatic extracts from *P. palmata* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars. (III)

The DPPH scavenging activity and ORAC of enzymatic extracts showed similar trend as TPC. The higher radical scavenging capacities of protease extracts could be linked to the improved extraction efficiency of phenolic compounds. Moreover, the liberation of LMW peptides and amino acids might also contribute to the enhanced scavenging activities (III).

A different pattern was observed for metal chelation. All the enzyme treatments had a negative impact on the chelating ability of the extracts. Algal dietary fibres are well known for their excellent metal binding capacity. The enzymatic degradation of these dietary fibres into oligosaccharides and simple sugars might reduce the chelating capacity to different extent, depending on the composition and structure of degradation products. There was no clear correlation between TPC and chelating capacity of *P. palmata* hydrolysates, supporting our previous finding that algal polyphenols were not effective metal chelators (II).

The relative contributions of crude polyphenol, crude polysaccharide and LMW aqueous fractions to the overall antioxidant activity of Umamizyme extract were studied. Crude polyphenol fraction showed the highest scavenging activity on both DPPH and peroxyl radicals, confirming the key role of algal polyphenols as primary, chain-breaking antioxidants (II). HMW polysaccharides contributed greatly to the ferrous ion-chelating and DPPH quenching activities of the hydrolysate. LMW peptides, amino acids as well as some of the mycosporine-like amino acids (MAAs) might be the major contributors for the moderate radical scavenging capacity of LMW aqueous fraction (Table 6, III).

Table 6. DPPH radical scavenging activity, ORAC and ferrous ion-chelating ability of crude polyphenol, polysaccharide and LMW aqueous fractions from Umamizyme extract of *P. palmata*. (III)

Sample	DPPH scavenging[a] (ARP)	ORAC[b] (μmol TE/g extract)	Fe^{2+} chelating ability (%)
Crude polyphenol fraction	5.4 ± 0.3	629.5 ± 15.2	57.2 ± 2.9[c]
Crude polysaccharide fraction	4.8 ± 0.3	102.9 ± 2.8	94.3 ± 1.8[c]
LMW aqueous fraction (<5 KDa)	2.4 ± 0.1	130.7 ± 3.4	20.5 ± 1.6[d]

Each value is expressed as means ± S.D. (n = 3).
[a] ARP, antiradical power (ARP=1/EC_{50}), EC_{50}: concentration of extract (mg/ml) required to scavenge 50% of the DPPH• in the reaction mixture.
[b] ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.
[c] was tested at concentration of 2.5 mg/ml.
[d] was tested at concentration of 5.0 mg/ml.

4.8 Enzyme treatment on the extraction of phlorotannins from *F. vesiculosus* and *L. hyperborea*

All the tested proteases and carbohydrases increased the amount of total extractable substances (TES) to different extent compared with water. However, enzyme treatments did not improve the recovery of phlorotannin compounds and the TPC of all the enzymatic extracts was lower than that of cold water (unpublished data). Accordingly, the antioxidant activities of *F. vesiculosus* extracts evaluated by DPPH· scavenging activity and ORAC were all lower in enzyme-assisted extraction system compared with water extraction (Figure 12 and unpublished data).

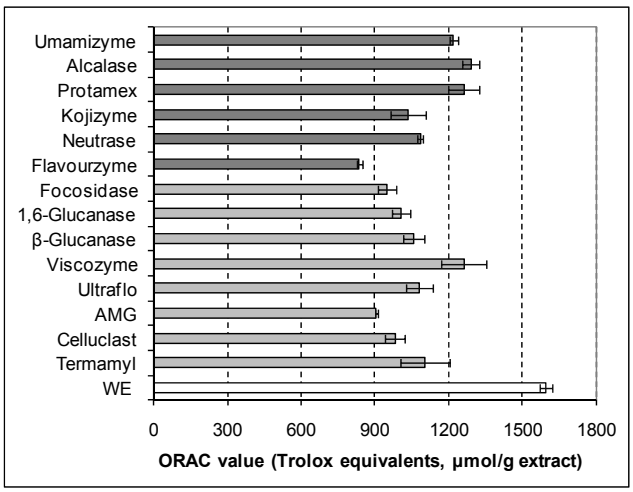


Figure 12. Oxygen radical absorbance capacity (ORAC) of different enzymatic extracts from *F. vesiculosus* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

The higher extraction yield but lower TPC and lower antioxidant activity of enzymatic extracts suggested that components other than phlorotannins were extracted and contributed to the yield. It can be assumed that the content of cell-wall-bound phlorotannins in *F. vesiculosus* is much lower than that of soluble phlorotannins. Therefore, the breakdown of HMW polysaccharides and proteins by these cell wall degrading enzymes contributed to higher yield without improvement of phlorotannin extraction and antioxidant activities of the hydrolysates. This finding is in accordance with a previous study by Koivikko *et al*. (2005), showing that the concentration of soluble phlorotannins in the cytoplasm of *F. vesiculosus* was around an order-of-magnitude higher than in the cell wall.

A similar trend was observed for *L. hyperborea*. The hydrolysis of the algae cell wall materials by these enzymes increased the TES but negatively impacted phlorotannin extraction and antioxidant activities (unpublished data). It could be inferred that no advantage could be gained in terms of commercial enzyme preparations to enhance the extraction of phlorotannins from this species.

4.9 Antioxidant effects of *F. vesiculosus* extract/fractions in fish model systems

In order to investigate the antioxidant performance of *F. vesiculosus* extract/fractions in fish model systems, to evaluate the contribution of phlorotannins to the overall antioxidant effect and to gain a basic understanding of how intrinsic antioxidant characteristics, molecular size may influence their antioxidant effectiveness, several extract and fractions of different purity, polarity and molecular size were chosen for model study. Crude 80% EtOH extract, phlorotannin-enriched EtOAc fraction, Sephadex subfraction LH–2 which was rich in phlorotannin oligomers and LH–5 which was rich in phlorotannin polymers were used in washed cod model. Instead of the 80% EtOH extract, two more subfractions LH–3 and LH–6 were tested in cod protein isolates.

4.9.1 Antioxidant effect in washed cod model–crude extract versus phlorotannin-enriched fraction

In washed cod model system, the addition of 80% EtOH extract and phlorotannin-enriched EtOAc fraction prolonged the lag time of both rancid odour and TBARS formation, but to different extent (Figure 13 (a) and (b), V). EtOAc fraction showed a greater inhibitory effect than the crude 80% EtOH extract, which could be attributed to its higher level of TPC.

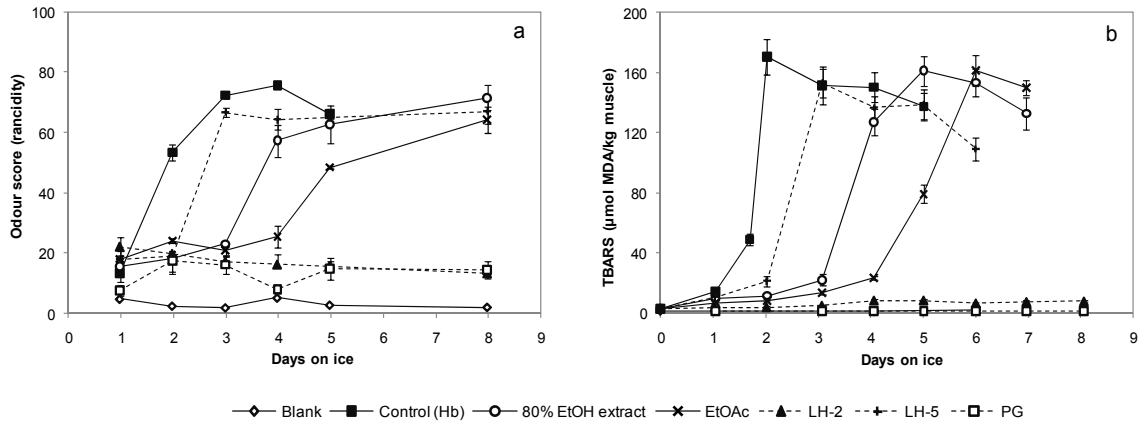


Figure 13. Effect of *F. vesiculosus* extract and fractions on the formation of (a) rancid odour, (b) TBARS in washed cod model. *F. vesiculosus* extract and fractions were added at 300 mg/kg model and propyl gallate (PG) was at 100 mg/kg model. Results are expressed as average ± standard deviation from duplicate samples. (V)

Therefore, the active components responsible for the inhibition of lipid oxidation in washed cod mince could be phlorotannins.

4.9.2 Antioxidant effect in washed cod model–phlorotannin oligomers versus polymers

Two subfractions (LH–2 and LH–5) derived from separation of the EtOAc fraction on Sephadex LH–20 column showed very different antioxidant activity in washed cod model system. The addition of the oligomeric subfraction (LH–2) was highly effective in preventing lipid oxidation and the formation of rancid odour and TBARS was complete inhibited throughout the entire storage period (Figure 13 (a) and (b), V). Its effectiveness at 300 mg/kg level was comparable to that of 100 mg/kg PG, which is known as one of the most effective antioxidants in muscle food systems. On the contrary, polymeric subfraction (LH–5) only exerted a slight retarding effect. The antioxidant potency of subfraction LH–2 was also supported by the slowest rate of redness loss (V).

4.9.3 Antioxidant effect in cod protein isolates–phlorotannin oligomers versus polymers

Owing to the different antioxidant behaviour of subfractions LH–2 and LH–5 observed in washed cod model, the main focus of cod protein isolate study was to compare the antioxidant effectiveness of various Sephadex subfractions.

Although different amount of membrane lipids can be removed during acid- and alkali-aided protein isolation, the residual membrane phospholipids still readily undergo oxidation in the presence of strong prooxidants like Hb, which limit their effective utilisation (Nolsøe and

Undeland, 2009). In this study, we observed that the process of Hb-mediated lipid oxidation of protein isolates was slower and less pronounced than in the washed cod model despite a similar fat content and fatty acid composition. In the control group with Hb, the maximum level of TBARS was only up to 40 μmol MDA/kg of sample after 3 days of ice storage (Figure 14, V). The slower oxidation rate of cod protein isolates could not be explained by the reduction of lipids after alkaline treatment or different amounts of susceptible polyunsaturated fatty acids. Previous studies have suggested that membrane phospholipids may become more buried and less accessible to pro-oxidants upon acid treatment followed by isoelectric precipitation (Kristinsson and Hultin 2004; Pazos *et al*., 2005; Vareltzis and Hultin, 2007). It is possible that a similar conformational change of the membranes also occurred at alkaline pH conditions, thus possibly explaining the difference between the isolate and washed muscle. Although the rate and extent of oxidation was less in protein isolates than washed cod muscle, the inhibition of lipid oxidation by different Sephadex subfractions showed a similar pattern as in washed cod model.

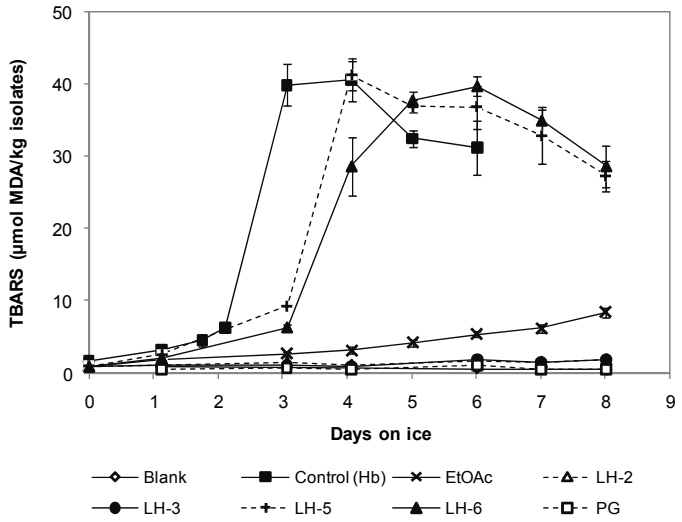


Figure 14. Effect of *F. vesiculosus* fractions on the formation of TBARS in alkali-solubilised cod muscle protein isolates. *F. vesiculosus* extract and fractions were added at 300 mg/kg model and propyl gallate (PG) was at 100 mg/kg model. Results are expressed as average ± standard deviation from duplicate samples. (V)

4.9.4 Partitioning behaviour–oligomer vs. polymers

The partitioning study showed that most of the phlorotannins in different Sephadex subfractions were more soluble in the water than in the oil phase. A simple or direct relationship could not be established between the polarity of the subfractions and their antioxidant potency in fish model systems, which reflects the complexity of the mechanism

involved in antioxidant protection in a heterogeneous fish matrix. (V). Because of the hydrophilic nature of phlorotannin oligomers, they are not likely to partition preferentially into the hydrophobic region of the membrane. Instead, they might be localised in close proximity to the membrane surface. Several studies have shown that directing antioxidants into muscle cell membranes can enhance the oxidative stability of muscle tissue (Ballesteros, 2009; Kathirvel and Richards, 2009; Raghavan and Hultin, 2004). But our study indicated that the antioxidant potency of phenolic antioxidants in fish model systems may not necessarily depend on their ability to penetrate into the membrane. Instead, the protective effect conferred by phlorotannin oligomers may be due to the antioxidant reactions occurring in the aqueous phase or other mechanisms that are not yet well understood.

4.9.5 Antioxidant mechanisms–oligomer vs. polymers?

The exact mechanisms accounting for the differential antioxidant effects of different phlorotannin components in fish models are not clear. One possible explanation can be that the small size and high polarity of phlorotannin oligomers in LH–2 and LH–3 may allow them to locate close to the polar surface of the membrane to effectively scavenge radicals generated in the aqueous phase. Therefore, phlorotannin oligomers may mainly act as the first line of defense by scavenging aqueous phase radicals before they attack the membranes, rather than as scavengers of chain-propagating lipid peroxyl radicals within the membrane. Several plant-derived polyphenols such as catechins and olive oil phenols were shown to regenerate α-tocopherol effectively (Mukai *et al*., 2005; Paiva-Martins *et al*., 2003). Similarly, phlorotannin oligomers may also have the capacity to regenerate the endogenous antioxidant α-tocopherol in fish muscle. On the other hand, the bulky size of highly polymeric phlorotannins in LH–5 and LH–6 may sterically hinder their interactions with the membrane bilayer, which could partly explain their lack of effect in both systems. In addition, these subfractions might also contain HMW complexes formed between phlorotannins and other macromolecules like proteins or carbohydrates. The complexation may greatly impair the ability of phlorotannins to protect membrane phospholipids from oxidation.

4.9.6 Can *in vitro* antioxidant tests predict the antioxidant effect in fish model system?

It was also of interest to examine whether chemical reaction-based antioxidant tests could be used to predict the antioxidant effect of phlorotannin antioxidants in complex fish systems. The results of the present study indicated that *in vitro* antioxidant tests did not consistently reflect the relative ability of phlorotannins to inhibit lipid oxidation in fish models.

Subfraction LH–2, which had the second highest TPC and reducing power and the strongest DPPH radical scavenging activity, completely prevented the initiation of lipid peroxidation in both washed cod mince and cod protein isolates (V). Interestingly, however, LH–5 with the third highest TPC level and considerable DPPH scavenging activity and reducing power (Table 4, Paper IV), showed a very weak inhibitory effect in both model systems (V). These results substantially reinforce the findings from early research that the ability of phenolic compounds to prevent lipid oxidation in food systems is determined not only by their inherent antioxidant properties, but also by other factors such as chemical structure, molecular size, location and orientation in targeted substrate as well as interactions with other food ingredients.

4.10 ROS scavenging activity of different subfractions in a mononuclear cell-based bioassay

In addition to potent antioxidant activity, algal polyphenols have been shown to possess interesting multifunctional properties. In this study, the ROS scavenging ability of *F. vesiculosus* subfractions was evauated in a monocyte-based cell model. All the subfractions separated by Sephadex LH–20 column and ultra-filtration exhibited potent *in vitro* ROS scavenging ability. At a concentration of 2.4 μg/ml, all the ultra-filtered subfractions showed a significantly high ability to scavenge ROS (generated by the stimulation of mononuclear cells) compared to the control (Figure 15, IV). The average ROS quenching ability appeared to increase with a decrease in MW of the subfractions, but the difference was not statistically

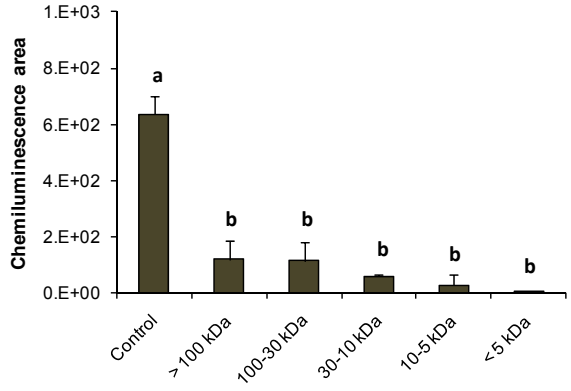


Figure 15. Effect of different MW subfractions of *F. vesiculosus* on PMA stimulated chemiluminescent emission of freshly prepared human mono-nuclear cells. The concentration of cells used for this assay was 5 x 10^5 cells/ ml. The reaction was started using 5 units of HRP. The samples were added at a final concentration of 2.4 μg/ ml of reaction mixture. (IV)

significant. However, the chemiluminescence quenching ability was not reflected in their DPPH radical scavenging activity, reducing power and iron chelating ability (IV).

4.11 Preliminary study on the ACE inhibitory activity

Some plant-derived polyphenols like tannins and procyanidins have been reported to possess ACE inhibitory activity (Actis-Goretta *et al*., 2003; Liu *et al*., 2003). However, little data are available regarding the effects of algal phlorotannins against ACE activity.

The inhibitory effect of three fractions from the 80% EtOH extract of *F. vesiculosus* increased with the increase of the concentration. The EtOAc-soluble fraction exhibited the highest ACE inhibitory activity with an IC_{50} value of 2.5 µg/ml, followed by *n*-butanol fraction (IC_{50} = 21.8 µg/ml), while the aqueous residue was far less effective (IC_{50} = 1166.1 µg/ml).

All the subfractions separated by Sephadex LH–20 column showed potent ACE inhibitory activity (Figure 16a). The highest level of inhibition was observed in subfractions LH–2 and LH–3 while the lowest was in LH–6. Further fractionation of EtOAc fraction by ultra-filtration afforded six subfractions with IC_{50} values ranging from 1.7 to 31.0 µg/ml (Figure 16b). The fractions with low MW were found to possess significantly higher activity than the high MW fractions.

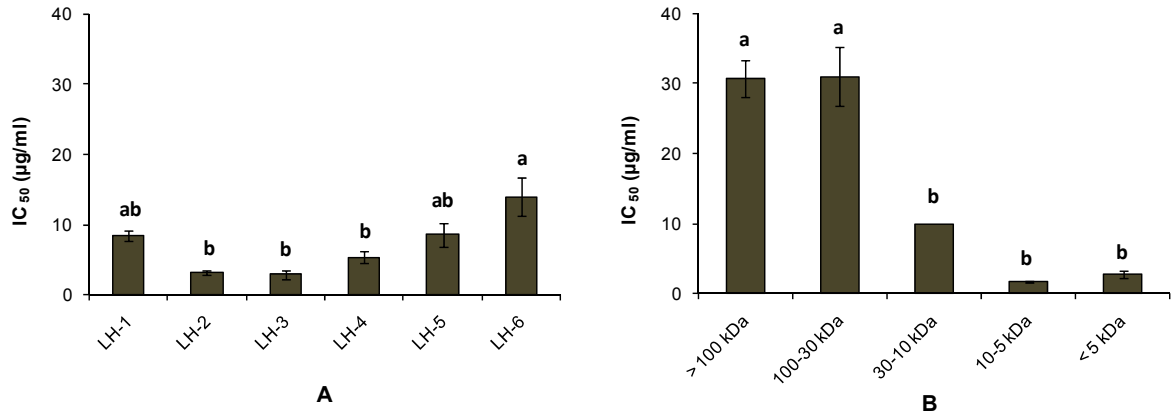


Figure 16. ACE inhibitory activity (IC_{50} value) of subfractions separated by Sephadex LH–20 column (A) and ultra-filtration (B) from EtOAc fraction of *F. vesiculosus*. Different letters in each figure indicate significant differences between the fractions ($p < 0.05$).

Although the mechanism of ACE inhibition by polyphenolic compounds has not been fully elucidated, the inhibitory effects of flavonoids and procyanidins has been suggested to be due to the generation of chelate complexes with the zinc atom within the active center of the angiotensin I-converting enzyme (Wagner *et al*., 1991). The relative potency seems to be

related to the number and arrangement of free hydroxyl groups in the polyphenol molecules, which has profound influence on their affinity for the enzyme and the number of available binding sites (Kang *et al*., 2003; Ottaviani *et al*., 2006). The impact of molecular size of phlorotannins is not clear, but the higher activity of low MW peptided than high MW peptides are well documented (Je *et al*., 2004; Raghavan and Kristinsson, 2009). The differential ACE inhibitory effects of various Sephadex and ultra-filtrated subfractions observed herein might be explained by their relative ability to interact with ACE. The low MW phlorotannin oligomers may have high affinity to the enzyme and possess the ability to bind to the active site of the ACE, whereas the bulky size of high MW phlorotannin polymers may sterically hinder their interactions with the enzyme. More detailed studies are needed to gain a better understanding of the mechanism of the action.

5 CONCLUSIONS

The studies provided better understanding of antioxidant properties of algal polyphenols (especially phlorotannins) derived from marine algae and the relative contribution of algal polyphenols to the overall antioxidant activity of seaweed extracts. The integrated use of *in vitro* antioxidant tests, mononuclear cell-based bioassay and fish model systems verified the complex mechanisms involved in the antioxidant effect of algal polyphenols in different systems and gave a more comprehensive antioxidant profile than a single test.

Various seaweed species collected from southwest coast of Iceland contained different levels of TPC and possessed varying degrees of antioxidant activity. Intertidal fucoid species (especially *Fucus vesiculosus* and *Fucus serratus*) with high level of TPC exhibited potent *in vitro* antioxidant activities. A phlorotannin-enriched fraction from *F. vesiculosus* had much higher radical scavenging capacity and reducing power than the other fractions and the original crude extract. In particular, all the subfractions separated by Sephadex LH–20 column and ultra-filtration showed very promising *in vitro* ROS scavenging activity. Further testing of the phlorotannin-containing extract and fractions in fish model systems demonstrated the potential application as natural antioxidants in fish products. The effectiveness of oligomeric subfractions at 300 mg/kg level was comparable to that of 100 mg/kg propyl gallate, which is known as one of the most effective antioxidants in muscle food systems. This fucoid species warrants further exploration as a viable source of natural antioxidants with broad potential applications in fish products, functional foods and nutraceuticals.

Protease treatment enhanced the extraction of water-soluble polyphenols and other antioxidant components from *P. palmata*. The improved radical scavenging capacities of protease extracts could be attributed to the improved extraction efficiency of phenolic compounds as well as the liberation of LMW peptides and amino acids. However, negative effects were observed in two brown algae, *F. vesiculosus* and *L. hyperborea*. Enzymatic treatments increased the extraction yield but negatively impacted on phlorotannin extraction and antioxidant activities. These results suggest that the effectiveness of enzyme-assisted extraction of polyphenols from seaweeds is species and enzyme-dependent. The merits and disadvantages of enzymatic extraction of phenolic antioxidants from a particular species should be carefully evaluated.

PCA and Pearson correlation analysis revealed that there were strong positive correlations between TPC, DPPH radical scavenging activity and ORAC of various seaweed extracts. On

the contrary, ferrous ion-chelating ability correlated neither with TPC, nor with DPPH or ORAC. In the enzyme-assisted extraction study, the crude polyphenol fraction derived from the Umamizyme extract of *P. palmata* possessed the highest peroxyl radical scavenging activity, whereas the crude polysaccharide fraction was more effective for chelating ferrous ions. Moreover, the DPPH scavenging and reducing activities were found to be positively correlated with the TPC among the crude extracts and different solvent fractions of *F. vesiculosus*. These studies clearly demonstrated that algal polyphenols mainly act as potent free radical scavengers and primary, chain-breaking antioxidants. Algal polyphenols do not appear to be very effective metal chelators as other algal constituents like HMW sulphated polysaccharides, proteins or peptides.

Careful selection of *in vitro* antioxidant tests based upon different reaction mechanisms help to understand the intrinsic antioxidant properties of seaweed extracts, which is very useful in initial antioxidant study. However, the chemical antioxidant activity did not consistently correlate with their relative ability to inhibit lipid oxidation in fish model systems. The antioxidant effectiveness of phlorotannins in heterogeneous fish muscle may not rely solely on the intrinsic reactivity toward radicals, but also on other factors such as the molecular size, location and orientation at the membrane interface, environmental conditions (e.g., temperature, pH and ionic strength) as well as interactions with other food ingredients.

The specific mechanisms by which these phlorotannin components prevented lipid oxidation in fish models have not been established. Several explanations have been put forward in this thesis to explain the differential antioxidant effects of oligomeric and polymeric phlorotannins. Owing to the small size and high polarity, phlorotannin oligomers are likely to be localised close to the polar surface of the membrane and effectively scavenge radicals generated in the aqueous phase before they attack the membranes. This may possibly indicate that phlorotannin oligomers act mainly as the first line of defense rather than as scavengers of chain-propagating lipid peroxyl radicals within the membrane. We suggest further that the antioxidant potency of phlorotannin antioxidants in fish model systems may not necessarily depend on their ability to penetrate into the membrane. The ability of oligomeric phlorotannins to regenerate the endogenous antioxidant α-tocopherol may be another possible mechanism. The poor activity of polymeric phlorotannin-rich subfractions may be attributed to their bulky size, which sterically hinders their interactions with the membrane bilayer. In addition, these subfractions might also contain HMW complexes formed between phlorotannins and other macromolecules like proteins or carbohydrates. The complexation may greatly impair the ability of phlorotannins to protect membrane phospholipids.

The studies provide a necessary basis and more importantly will stimulate future research to enhance and broaden the value add utilisation of seaweeds. Seaweed derived phlorotannins showed great potential for future development of novel fish products with enhanced oxidative stability and nutritional value. The preliminary results also suggested the potential applications of phlorotannins in the formulation of functional foods and nutraceuticals.

6 FUTURE PERSPECTIVES

The application of new packaging and chilling preservation techniques and the addition of functional and bioactive ingredients is of great importance for the maintenance of the prime quality and the storage stability of seafood based products. The use of seaweed derived polyphenol antioxidants may offer a new approach to enhance their oxidative stability and nutritional value.

Marine algae are an attractive source of natural antioxidants with remarkable and diverse health-promoting properties. The results from the studies clearly showed that phlorotannins are one of the most active ingredients responsible for the superior antioxidant activity of *F. vesiculosus*. The antioxidant effects of *F. vesiculosus* extract and fractions in fish model systems suggest the great potential of phlorotannin compounds as natural antioxidants in fish and fish products. The potent ROS quenching and ACE inhibitory activities demonstrate the additional health promoting effects of phlorotannins. These findings warrant further investigations to explore their potential nutraceutical and pharmaceutical applications.

The antioxidant effects of algal polyphenols in food systems have rarely been evaluated. More studies are necessary to investigate the efficacy of phlorotannin antioxidants in different food systems and the stability during food processing and storage. Other aspects which may influence their application in food products should also be investigated. Phlorotannins derived from brown algae generally have an unpleasant bitter or pungent taste and distinct colour, which may negatively affect the sensory quality of food products. The interactions between phlorotannin compounds and proteins may modify the functional properties of proteins and influence the bioavailability of both proteins and phlorotannins. All these issues are of critical importance for future food applications.

In order to direct seaweed based antioxidants into different food products or supplements for human consumption, the extraction and purification techniques are of prime importance. Solvent extraction and fractionation were shown to be effective for the extraction and separation of phlorotannin components from *F. vesiculosus* in the present study. But the extraction solvent must be adequately selected to meet the requirements set by EU regulations. Due to the increased public awareness and the growing environmental and safety concerns of using large amounts of some organic solvents in the production of food ingredients, it is necessary to develop effective and environmentally friendly technologies for the extraction, separation and purification of algal polyphenols, including supercritical fluid extraction, subcritical water extraction, enzyme-assisted extraction and membrane separation system.

Further optimisation and scale-up of these new technologies is crucial for successful commercial developments. Enzyme-assisted extraction can be an alternative extraction method for some seaweed species, but further studies are needed to gain a better understanding of the distribution pattern of phenolic compounds in algal tissue, the structure and organisation of algal cell walls, the mechanism of the interaction between enzyme and target substrate in algal tissue as well as the kinetics and mechanisms of enzyme-enhanced liberation of phenolic compounds from different seaweed species.

The use of a combination of methodology based on different mechanisms is recommended to build a more complete picture of the antioxidant capacity of algal polyphenols. The antioxidant effect of phlorotannins is system dependant and it is important to conduct studies in appropriate models which closely mimic the targeted food system. Washed fish mince model has the advantage of mimicking the main features of fish muscle and can provide useful information for predicting the antioxidant effect of algal polyphenols in real fish systems. It must be stressed however that washed fish mince model also has its limitations. The mechanisms of oxidative deterioration in fish muscle are very complex. Different prooxidative systems such as lipoxygenase (LOX) and haemeproteins act cooperatively to initiate the rapid lipid oxidation. Hb-initiated lipid oxidation in washed fish mince model does not reflect other mechanisms involved in the oxidation of real fish muscle, for example, the important role of LOXs and the cooperative actions of different prooxidative systems. Therefore, further studies are needed to investigate the efficiency of phlorotannins towards other oxidative processes in fish muscle. The antioxidant effects of algal polyphenols also need to be further confirmed in real fish systems.

The results from the present and previous studies have shown that brown algal phlorotannins possess extraordinary antioxidant and other bioactive properties. However, studies of seaweed derived antioxidants are still in their infancy and continued research efforts are needed in order to develop this underutilised resource into a food grade antioxidant or dietary food supplement. Purification, characterisation and structural analysis of phlorotannin compounds need to be performed to gain a better understanding of exact mechanisms involved in the antioxidant protection and the structure-antioxidant activity relationship. Well designed *in vivo*, animal and human clinical studies should be carried out to systematically evaluate their health benefits and potential risks. The impact of processing conditions on the stability and bioavailability of algal polyphenols needs to be investigated. The adverse interaction or complexation of polyphenolic compounds with other food ingredients (proteins,

carbohydrates and lipids) as well as the possible formation of toxic, allergenic or carcinogenic substances should also be addressed.

More work is needed to understand their bioavailability *in vivo*. The stability during gastrointestinal digestion, the absorption in the gastrointestinal tract as well as the effects of metabolic transformation and degradation on the antioxidant activity of algal polyphenols warrant further investigation.

7 ACKNOWLEDGEMENTS

Most of this study was carried out at Matís - Icelandic Food Research. The work was financed by AVS R&D Fund of Ministry of Fisheries in Iceland, Icelandic Research Fund and Integrated EU Project SEAFOODplus-Project 2.2 SEAFOODSENSE. This study is in collaboration with Hollusta úr hafinu ehf. (now Íslensk hollusta ehf.).

First of all, I would like to express my gratitude to my supervisor, Dr. Gudrún Ólafsdóttir, for her wise supervision, encouragement, friendship and motivation at all stages of my study. Also for endless patience and help during the most challenging part of my dissertation.

My doctoral committee members: Guðjón Þorkelsson, Hörður G. Kristinsson, Charlotte Jacobsen and Guðmundur Óli Hreggviðsson for elaborate guidance, invaluable advice, support and encouragement throughout my doctoral study. Hörður G. Kristinsson motivated and encouraged me to write the two book chapters, which have led me into more indepth understanding of the amazing bioactive properties of seaweeds.

United Nations University-Fisheries Training Programme for providing a PhD scholarship and all financial support throughout the course of my study. I would like to address special thanks to Dr. Tumi Tómasson and Mr. Thor H. Asgeirsson. Their continuous support, guidance and direction were crucial for accomplishing my project. Sigridur Kr. Ingvarsdóttir for warmly encourages and always taking care of me during my stay in Iceland. All the UNU-FTP fellows for their support and many enjoyable times during these years.

Matís (Icelandic Food Research) for providing the opportunity to carry out this study. The Division of Biotechnology and Biomolecules (Prokaria) has provided me excellent environment and facilities to work and learn. The staff at Matís is thanked for their valued contribution in sensory, chemical and microbial analysis. Special thanks to Kolbrún Sveinsdóttir, Hannes Magnússon, Emilía Martinsdóttir and Hélène L. Lauzon, for their guidance, support and help at the beginning of this study.

I would like to express my hearty thanks to Rósa Jónsdóttir for good collaboration, support, valuable help on GC and statistic analysis and contribution to manuscript writing. Jón Óskar Jónsson has always been there when needed in the laboratory. My colleagues and friends

Patricia Yuca Hamaguchi and Annabelle Vrac for their contributing to ACE and GC data and fruitful discussion. Margrét Geirsdóttir for her good advice and help.

Special thanks to Karl Gunnarsson at Marine Research Institute for identification of the seaweed species and beautiful seaweed photos.

Sivakumar Raghavan at the Department of Food Science and Human Nutrition, University of Florida, USA, for his skilful help in mononuclear cell model, also for good discussion and advice.

Isabel Medina and María Jesús Gonzalez at Instituto de Investigaciones Marinas del CSIC, Vigo, Spain, for good collaboration and help in HPLC-DAD-MASS analysis.

My good friends Xiaojie Nie and Bingcui Qi have been so supportive and have taken care of me for the past few years, making the life in Iceland unforgettable.

Finally, I would like to thank my parents, MaoZhong Wang and Manquan Pang, my husband Jun Zhuang. Their endless love, support, patience and understanding played a decisive role in accomplishing this study. My daughter Yixue Zhuang for being the light of my life.

8 REFERENCES

Actis-Goretta, L., Ottaviani, J. I., Keen, C. L., & Fraga, C. G. (2003). Inhibition of angiotensin converting enzyme (ACE) activity by flavan-3-ols and procyanidins. *FEBS Letters*, 555(3), 597-600.

Ahn, G. N., Kim, K. N., Cha, S. H., Song, C. B., Lee, J., Heo, M. S., et al. (2007). Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H_2O_2-mediated DNA damage. *European Food Research and Technology*, 226(1-2), 71-79.

Almajano, M. P., & Gordon, M. H. (2004). Synergistic effect of BSA on antioxidant activities in model food emulsions. *Journal of the American Oil Chemists' Society*, 81(3), 275-280.

Altunkaya, A., Becker, E. M., Gökmen, V., & Skibsted, L. H. (2009). Antioxidant activity of lettuce extract (*Lactuca sativa*) and synergism with added phenolic antioxidants. *Food Chemistry*, 115(1), 163-168.

Anggadiredja, J., Andyani, R., Hayati, & Muawanah. (1997). Antioxidant activity of *Sargassum polycystum* (Phaeophyta) and *Laurencia obtusa* (Rhodophyta) from Seribu Islands. *Journal of Applied Phycology*, 9(5), 477-479.

Appel, H. M., Govenor, H. L., D'Ascenzo, M., Siska, E., & Schultz, J. C. (2001). Limitations of Folin assays of foliar phenolics in ecological studies. *Journal of Chemical Ecology*, 27(4), 761-778.

Asp, N.G., Bryngelsson, S. (2008). Health Claims in Europe: New Legislation and PASSCLAIM for Substantiation. *Journal of Nutrition*, 138(12), 1210S–1215S.

Athukorala, Y., Kim, K. N., & Jeon, Y. J. (2006). Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food and Chemical Toxicology*, 44(7), 1065-1074.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), 191-203.

Ballesteros, A. T. (2009). Antioxidant distribution and effectiveness in a model muscle system. University of Massachusetts Amherst. Ph.D. Thesis. pp. 30-57.

Banias, C., Oreopoulou, V., & Thomopoulos, C. D. (1992). The Effect of primary antioxidants and synergists on the activity of plant-extracts in lard. *Journal of the American Oil Chemists' Society*, 69(6), 520-524.

Baron, C. P., & Andersen, H. J. (2002). Myoglobin-induced lipid oxidation: a review. *Journal of Agricultural and Food Chemistry*, 50(14), 3887-3897.

Baron, C. P., Skebsted, L. H., & Andersen, H. J. (1997). Prooxidative activity of myoglobin species in linoleic acid emulsions. *Journal of Agricultural and Food Chemistry*, 45(5), 1704-1710.

Becker, E. M., Nissen, L. R., & Skibsted, L. H. (2004). Antioxidant evaluation protocols: Food quality or health effects. *European Food Research Technology*, 219(6), 561-571.

Becker, E. M., Ntouma, G., & Skibsted, L. H. (2007). Synergism and antagonism between quercetin and other chain-breaking antioxidants in lipid systems of increasing structural organisation. *Food Chemistry*, 103(4), 1288-1296.

Benjakul, S., Visessanguan, W., Phongkanpai, V., & Tanaka, M. (2005). Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. *Food Chemistry*, 90(1-2), 231-239.

Cadwallader, K. R. (2000). Enzymes and flavor biogenesis in fish. In N. F. Haard & B. K. Simpson (Ed.), *Seafood enzymes: utilization and influence on postharvest seafood quality*. (pp. 365-383). New York: Marcel Dekker, Inc.

Canadanovic-Brunet, J. M., Djilas, S. M., Cetkovic, G. S., Tumbas, V. T., Mandic, A. I., & Canadanovic, V. M. (2006). Antioxidant activities of different *Teucrium montanum* L. extracts. *International Journal of Food Science and Technology*, 41(6), 667-673.

Cerantola, S., Breton, F., Ar Gall, E., & Deslandes, E. (2006). Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. *Botanica Marina*, 49(4), 347-351.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT - Food Science and Technology*, 41(6), 1067-1072.

Chun, O. K., & Kim, D. O. (2004). Consideration on equivalent chemicals in total phenolic assay of chlorogenic acid-rich plums. *Food research international*, 37(4), 337-342.

Codex Alimentarius (2004). Codex Guidelines for Use of Nutritional and Health Claims. ALINORM 04/27/41, Codex Committee on Food Labeling.

Connan, S., Goulard, F., Stiger, V., Deslandes, E., & Ar Gall, E. (2004). Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. *Botanica Marina*, 47(5), 410-416.

Davalos, A., Gomez-Cordoves, C., & Bartolome, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry*, 52(1), 48-54.

Davidson, C. M., & Cronin, F. (1973). Medium for the selective enumeration of lactic acid bacteria from foods. *Applied Microbiology*, 26(3), 439-440.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effecticeness in food. *Journal of Agricultural and Food Chemistry*, 53(10), 4303-4310.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674-677.

Duan, X. J., Zhang, W. W., Li, X. M., & Wang, B. G. (2006). Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata. Food Chemistry*, 95(1), 37-43.

Duh, P. D. (1998). Antioxidant activity of burdock (Arctium lappa Linne): its scavenging effect on free-radical and active oxygen. *Journal of the American Oil Chemist's Society*, 75(4), 455–461.

Elisia, I., Hu, C., Popovich, D. G., & Kitts, D. D. (2007). Antioxidant assessment of an anthocyanin-enriched blackberry extract. *Food Chemistry,* 101(3), 1052-1058.

Everse, J., & Hsia, N. (1997). The toxicities of native and modified hemoglobins. *Free Radical Biology and Medicine*, 22(6), 1075-1099.

Foegeding, E. A., Lanier, T. C., & Hultin, H. O. (1996). Characteristics of edible muscle tissues. In: O. R. Fennema (Ed.), *Food chemistry* (pp. 879-942). New York: Marcel Dekker.

Foti, M. C., Daquino, C., & Geraci, C. (2004). Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH• radical in alcoholic solutions. *Journal of Organic Chemistry*, 69(7), 2309-2314.

Frankel, E. N. (1998). *Lipid Oxidation*. Dundee, Scotland: The Oily Press Ltd.

Frankel, E. N. (2005). *Lipid oxidation*. Bridgwater, England: The Oily Press Ltd.

Frankel, E. N. (2007). *Antioxidants in Food and Biology: Facts and Fiction*. Bridgewater, England: The Oily Press.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, 80(13), 1925-1941.

Fu, X. J., Xu, S. Y., & Wang, Z. (2009). Kinetics of lipid oxidation and off-odor formation in silver carp mince: The effect of lipoxygenase and haemoglobin. *Food Research International*, 42(1), 85-90.

Galland-Irmouli, A. V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., et al. (1999). Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse). *Journal of Nutritional Biochemistry*, 10(6), 353-359.

Glombitza, K. W., & Pauli, K. (2003). Fucols and phlorethols from the brown alga *Scytothamnus australis* Hook. *et* Harv. (Chnoosporaceae). *Botanica Marina*, 46(3), 315-320.

Gomez-Guillen, M. C., & Montero, M. P. (2007). Polyphenol uses in seafood conservation. *American Journal of Food Technology*, 2(7), 593-601.

Gordon, M. H. (1990). The mechanism of antioxidant action *in vitro*. In B. J. F. Hudson (Ed.), *Food antioxidants* (pp. 1-18). London, UK: Elsevier Applied Science.

Gram, L., Trolle, G., & Huss, H. H. (1987). Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. *International Journal of Food Microbiology*, 4(1), 65-72.

Graversen, H. B., Becker, E. M., Skibsted, L. H., & Andersen, M. L. (2008). Antioxidant synergism between fruit juice and alpha-tocopherol. A comparison between high phenolic black chokeberry (*Aronia melanocarpa*) and high ascorbic blackcurrant (*Ribes nigrum*). *European Food Research and Technology*, 226(4), 737-743.

Grunwald, E. W., & Richards, M. P. (2006). Mechanisms of heme protein-mediated lipid oxidation using hemoglobin and myoglobin variants in raw and heated washed muscle. *Journal of Agricultural and Food Chemistry*, 54(21), 8271-8280.

Gunnarsson, G., Undeland, I., Sannaveerappa, T., Sandberg, A. S., Lindgard, A., Mattsson-Hulten, L., et al. (2006). Inhibitory effect of known antioxidants and of press juice from herring (*Clupea harengus*) light muscle on the generation of free radicals in human monocytes. *Journal of Agricultural and Food Chemistry*, 54(21), 8212-8221.

Hagerman, A. E. (1988). Extraction of tannin from fresh and preserved leaves. *Journal of Chemical Ecology*, 14(2), 453-461.

Halliwell, B. (1997). Antioxidants in disease mechanisms and therapy. In H. Sies (Ed.), *Advances in Pharmacology* (Vol. 38, pp. 3–17). New York, NY: Academic Press.

Han, T. J., & Liston, J. (1987). Lipid peroxidation and phospholipid hydrolysis in fish muscle microsomes and frozen fish. *Journal of Food Science*, 52(2), 294-296.

Hargrove, M. S., Wilkinson, A. J., & Olson, J. S. (1996). Structural factors governing hemin dissociation from metmyoglobin. *Biochemistry*, 35(35), 11300-11309.

He, Y. H., & Shahidi, F. (1997). Antioxidant activity of green tea and its catechins in a fish meat model system. *Journal of Agricultural and Food Chemistry*, 45(11), 4262-4266.

Hemat, R. A. S. (2007). Fat and muscle dysfunction. In R. A. S. Hemat (Ed.), *Andropathy* (pp. 83-85). Dublin, Ireland: Urotext.

Hemmi, A., Makinen, A., Jormalainen, V., & Honkanen, T. (2005). Responses of growth and phlorotannins in *Fucus vesiculosus* to nutrient enrichment and herbivory. *Aquatic Ecology*, 39(2), 201-211.

Heo, S. J., Lee, K. W., Song, C. B., & Jeon, Y. J. (2003). Antioxidant activity of enzymatic extracts from brown seaweeds. *Algae*, 18(1)**,** 71-81.

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology*, 96(14), 1613-1623.

Honzel, D., Carter, S. G., Redman, K. A., Schauss, A. G., Endres J. R., & Jensen, G. S. (2008). Comparison of chemical and cell-based antioxidant methods for evaluation of foods and natural products: generating multifaceted data by parallel testing using erythrocytes and polymorphonuclear cells. *Journal of Agricultural and Food Chemistry*, 56(18), 8319-8325.

Hras, A. R., Hadolin, M., Knez, Z., & Bauman, D. (2000). Comparison of antioxidative and synergistic effects of rosemary extract with alpha-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chemistry*, 71(2), 229-233.

Hsieh, R. J., & Kinsella, J. E. (1989). Lipoxygenase generation of specific volatile flavor carbonyl compounds in fish tissues. *Journal of Agricultural and Food Chemistry*, 37(2), 279-286.

Huang, D. J., Ou, B. X., Hampsch-Woodill, M., Flanagan, J. A. & Deemer, E. K. (2002). Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidant using randomly methylated *β*-cyclodextrin as the solubility enhancer. *Journal of Agricultural and Food Chemistry*, 50(7), 1815-1821.

Huang, D. J., Ou, B. X., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, 53(6), 1841-1856.

IFT Expert Report (2005). Functional Foods: Opportunities and Challenges. Chicago, Illinois, USA, the Institute of Food Technologists.

Iwai, K. (2008). Antidiabetic and antioxidant effects of polyphenols in brown alga *Ecklonia stolonifera* in genetically diabetic KK-A^y mice. *Plant Foods for Human Nutrition*, 63(4), 163-169.

Je, J. Y., Park, P. J., Kwon, J. Y., & Kim, S. K. (2004). A novel angiotensin I converting enzyme inhibitory peptide from Alaska Pollack (*Theragra chalcogramma*) frame protein hydrolysate. *Journal of Agricultural and Food Chemistry*, 52(26), 7842-7845.

Jerez, M., Tourino, S., Sineiro, J., Torres, J. L., & Nunez, M. J. (2007). Procyanidins from pine bark: Relationships between structure, composition and antiradical activity. *Food Chemistry*, 104(2), 518-527.

Jónsdóttir, R., Bragadóttir, M., & Ólafsdóttir, G. (2007). The role of volatile compounds in odor development during hemoglobin-mediated oxidation of cod muscle membrane lipids. *Journal of Aquatic Food Product Technology*, 16(4), 67-86.

Jormalainen, V. & Honkanen, T. (2004). Variation in natural selection for growth and phlorotannins in the brown alga *Fucus vesiculosus*. *Journal of Evolutionary Biology*, 17(4), 807-820.

Josephson, D. B., Lindsay, R. C., Stuiber, D. A. (1984). Biogenesis of lipid-derived volatile aroma compounds in the emerald shiner (*Notropis atherinoides*). *Journal of Agricultural and Food Chemistry*, 32(6), 1347-1352.

Kallithraka, S., Garcia-Viguera, C., Bridle, P., & Bakker, J. (1995). Survey of solvents for the extraction of grape seed phenolics. *Phytochemical Analysis,* 6(5), 265-267.

Kang, D. G., Lee, Y. S., Kim, H. J., Lee, Y. M., & Lee, H. S. (2003). Angiotensin converting enzyme inhibitory phenylpropanoid glycosides from *Clerodendron trichotomum*. *Journal of Ethnopharmacology*, 89(1), 151-154.

Kantz, K., & Singleton, V. L. (1990). Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *American Journal of Enology and Viticulture*, 41(3), 223-228.

Karawita, R., Siriwardhana, N., Lee, K. W., Heo, M. S., Yeo, I. K., Lee, Y. D., et al. (2005). Reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition properties of different solvent fractions from *Hizikia fusiformis*. *European Food Research and Technology*, 220(3-4), 363-371.

Kathirvel, P., & Richards, M. P. (2009). Mechanisms by which flavonol aglycones inhibit lipid oxidation better than glycosylated flavonols in comminuted muscle tissue. *Food Chemistry*, 117(1), 75-82.

Koivikko, R. (2008). Brown algal phlorotannins. University of Turku, Finland. Ph.D. Thesis. pp. 34-38.

Koivikko, R., Loponen, J., Honkanen, T., & Jormalainen, V. (2005). Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *Journal of Chemical Ecology*, 31(1), 195-212.

Koivikko, R., Loponen, J., Pihlaja, K., & Jormalainen, V. (2007). High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. *Phytochemical Analysis*, 18(4), 326-332.

Kristinsson, H. G., & Hultin, H. O. (2004). The effect of acid and alkali unfolding and subsequent refolding on the pro-oxidative activity of trout hemoglobin. *Journal of Agricultural and Food Chemistry*, 52(17), 5482-5490.

Kristinsson, H. G., Theodore, A. E., Demir, N., & Ingadottir, B. (2005). A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. *Journal of Food Science*, 70(4), C298-306.

Kruger, C. L., & Mann, S. W. (2003). Safety evaluation of functional ingredients. *Food and Chemical Toxicology*, 41(6), 793-805.

Kubow, S. (1992). Routes of formation and toxic consequences of lipid oxidation products in foods. *Free Radical Biology and Medicine*, 12(1)**,** 63-81.

Kumaran, A., & Karunakaran, R. J. (2006). Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*. *Food Chemistry*, 97(1), 109-114.

Lee, S., Faustman, C., Djordjevic, D., Faraji, H., & Decker, E. A. (2006a). Effect of antioxidants on stabilization of meat products fortified with n-3 fatty acids. *Meat Science*, 72(1), 18-24.

Lee, C. H., Krueger, C. G., Reed, J. D., & Richards, M. P. (2006b). Inhibition of hemoglobin-mediated lipid oxidation in washed fish muscle by cranberry components. *Food Chemistry*, 99 (3), 591-599.

Liu, J. C., Hsu, F. L., Tsai, J. C., Chan, P., Liu, J. Y. H., & Thomas, G. N. *et al.* (2003). Antihypertensive effects of tannins isolated from traditional Chinese herbs as non-specific inhibitors of angiotensin converting enzyme. *Life Sciences*, 73(12), 1543-1555.

Ma, Y. T., & Cheung, P. C. K. (2007). Spectrophotometric determination of phenolic compounds by enzymatic and chemical methods-A comparison of structure-activity relationship. *Journal of Agricultural and Food Chemistry*, 55(10), 4222-4228.

Magalhães, L. M., Santos, M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2009), Flow injection based methods for fast screening of antioxidant capacity. *Talanta*, 77(5), 1559-1566.

Magalhães, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, 613(1), 1-19.

Malle, P., & Poumeyrol, M. (1989). A new chemical criterion for the quality control of fish: trimetylamine/total volatile basic nitrogen (%). *Journal of Food Protection*, 52(6), 419-423.

Manning, J. M., Dumoulin, A., Li, X. F., & Manning, L. R. (1998). Normal and abnormal protein subunit interactions in hemoglobins. *Journal of Biological Chemistry*, 273(31), 19359-19362.

McDonald, R. E. & Hultin, H. O. (1987). Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. *Journal of Food Science*, 52(1), 15-21.

Medina, I., Saeed, S., & Howell, N. (1999). Enzymatic oxidative activity in sardine (*Sardina pilchardus*) and herring (*Clupea harengus*) during chilling and correlation with quality. *European Food Research and Technology*, 210(1), 34-38.

Moore, J., & Yu, L. (2008). Method for antioxidant capacity estimation of wheat and wheat-based food products. In L.Yu (Ed.), *Wheat Antioxidants* (pp. 118-172). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Mukai, K., Mitani, S., Ohara, K., & Nagaoka, S. I. (2005). Structure-activity relationship of the tocopherol-regeneration reaction by catechins. *Free Radical Biology and Medicine*, 38(9), 1243-1256.

Nagai, T., & Yukimoto, T. (2003). Preparation and functional properties of beverages from sea algae. *Food Chemistry*, 81(3), 327-332.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I., & Nakamura, T. (2002). Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *Journal of Antimicrobial Chemotherapy*, 50(6), 889-893.

Nakai, M., Kageyama, N., Nakahara, K., & Miki, W. (2006). Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum*. *Marine Biotechnology*, 8(4), 409-414.

Nakamura, T., Nagayama, K., Uchida, K., & Tanaka, R. (1996). Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fisheries Science*, 62(6), 923- 926.

Nawar, W. W. (1996). Lipids. In: O. R. Fennema (Ed.), *Food chemistry* (3rd ed.) (pp. 225–319). New York: Marcel Dekker.

Nolsøe, H., & Undeland, I. (2009). The acid and alkaline solubilisation process for the isolation of muscle proteins: state of the art. *Food and Bioprocess Technology*, 2(1), 1-27.

Olafsdottir, G., Lauzon, H. L., Martinsdottir, E., Oehlenschläger, J., & Kristbergsson, K. (2006). Evaluation of shelf-life of superchilled cod (*Gadus morhua*) fillets and the influence of temperature fluctuations during storage on microbial and chemical quality indicators. *Journal of Food Science*, 71(2), S97-109.

Ottaviani, J. I., Actis-Goretta, L., Villordo, J. J., & Fraga, C. G. (2006). Procyanidin structure defines the extent and specificity of angiotensin I converting enzyme inhibition. *Biochimie*, 88(3-4), 359-365.

Ou, B. X., Huang, D. J., Hampsch-Woodill, M., Flanagan, J. A., & Deemer, E. K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *Journal of Agricultural and Food Chemistry*, 50(11), 3122-3128.

Paiva-Martins, F., Gordon, M. H., & Gameiro, P. (2003). Activity and location of olive oil phenolic antioxidants in liposomes. *Chemistry and Physics of Lipids*, 124(1), 23-36.

Pan, B. S., & Kuo, J. M. (2000). Lipoxygenases. In N. F. Haard & B. K. Simpson (Ed.), *Seafood enzymes: utilization and influence on postharvest seafood quality*. (pp. 317-336). New York: Marcel Dekker, Inc.

Pavia, H., & Toth, G. B. (2000). Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. *Hydrobiologia*, 440(1-3), 299-305.

Pazos, M., Medina, I., Hultin, H. O. (2005). Effect of pH on hemoglobin-catalyzed lipid oxidation in cod muscle membranes in vitro and in situ. *Journal of Agricultural and Food Chemistry*, 53(9), 3605-3612.

Pedrielli, P., & Skibsted, L. H. (2002). Antioxidant synergy and regeneration effect of quercetin, (−)-epicatechin, and (+)-catechin on α-tocopherol in homogenous solutions of peroxidating methyl linoleate. *Journal of Agriculture and Food Chemistry*, 50(24), 7138-7144.

Peyrat-Maillard, M. N., Cuvelier, M. E., & Berset, C. (2003). Antioxidant activity of phenolic compounds in 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation: synergistic and antagonistic effects. *Journal of the American Oil Chemists' Society*, 80(10), 1007-1012.

Pinelo, M., Ruiz-Rodríguez, A., Sineiro, J., Señoráns, F. J., Reglero, G., & Núñez, M. J. (2007). Supercritical fluid and solid–liquid extraction of phenolic antioxidants from grape pomace: a comparative study. *European Food Research and Technology*, 226(1-2), 199-205.

Plaza, M., Cifuentes, A., & Ibanez, E. (2008). In the search of new functional food ingredients from algae. *Trends in Food Science & Technology*, 19(1), 31-39.

Pokorny, J., & Sakurai, H. (2002). Role of oxidized lipids in nonenzymic browning reactions. *International Congress Series*, 1245, 373-374.

Prior, R. L., & Cao, G. H. (1999). In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radical Biology and Medicine*, 27(11-12), 1173-1181.

Prior, R. L., Wu, X. L., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements**.** *Journal of Agricultural and Food Chemistry*, 53(10), 4290-4302.

Ragan, M. A., & Glombitza, K. W. (1986). Phlorotannins, brown algal polyphenols. In F. E., Round, & D. J., Chapman (Ed.), *Progress in Phycological Research*, (pp. 129-241). Bristol, UK: Biopress Ltd.

Raghavan, S., & Hultin, H. O. (2004). Distribution of exogenous delta-tocopherol between the membrane lipids and triacylglycerols of a cod muscle-triacylglycerol model system. *Journal of Agricultural and Food Chemistry*, 52(20), 6294-6299.

Raghavan, S., Kristinsson, H. G. (2009). ACE-inhibitory activity of tilapia protein hydrolysates. *Food Chemistry*, 117(4), 582-588.

Raghavan, S., Kristinsson, H. G., & Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of tilapia (*Oreochromis niloticus*) protein hydrolysates. *Journal of Agricultural and Food Chemistry*, 56(21), 10359-10367.

Regulation (EC) No. 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. *Official Journal of the European Union, L 404*, 9-25. Brussels: European Parliament and Council of the European Union.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine,* 20(7), 933-956.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, 2(4), 152-159.

Richards, M. P., & Hultin, H. O. (2000). Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *Journal of Agricultural and Food Chemistry*, 48(8), 3141-3147.

Richards, M. P., & Hultin, H. O. (2003). Effects of added hemolysates from mackerel, herring and trout on lipid oxidation of washed cod muscle. *Fisheries Science*, 69(6), 1298-1300.

Richards, M. P., Modra, A. M., & Li, R. (2002). Role of deoxyhemoglobin in lipid oxidation of washed cod muscle mediated by trout, poultry and beef hemoglobins. *Meat Science,* 62(2), 157-163.

Roberts, W. G., & Gordon, M. H. (2003). Determination of the total antioxidant activity of fruits and vegetables by a liposome assay. *Journal of Agriculture and Food Chemistry*, 51(5), 1486-1493.

Sanchez-Alonso, I., Borderias, J., Larsson, K., & Undeland, I. (2007). Inhibition of hemoglobin-mediated oxidation of regular and lipid-fortified washed cod mince by a white grape dietary fiber. *Journal of Agricultural and Food Chemistry*, 55(13), 5299-5305.

Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 76(2), 270-276.

Sannaveerappa, T., Sandberg, A. S. & Undeland, I. (2007). Evaluation of occasional nonresponse of a washed cod mince model to hemoglobin (Hb)-mediated oxidation. *Journal of Agricultural and Food Chemistry*, 55(11), 4429-4435.

Santoso, J., Yoshie, Y., & Suzuki, T. (2002). The distribution and profile of nutrients and catechins of some Indonesian seaweeds. *Fisheries Science*, 68 (Suppl.), 1647-1648.

Santoso, J., Yoshie-Stark, Y., & Suzuki, T. (2004). Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. *Fisheries Science*, 70(1), 183-188.

Schaich, K. (2008). Choosing antioxidants for food and medical applications. http://www.ingredientsdirectory.com/reports/report1.pdf.

Schoenwaelder, M. E. A. (2002). The occurrence and cellular significance of physodes in brown algae. Phycologia, 41(2), 125-139.

Senevirathne, M., Kim, S. H., Siriwardhana, N., Ha, J. H., Lee, K. W., & Jeon, Y. J. (2006). Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. *Food Science and Technology International*, 12(1), 27-38.

Shahidi, F., & Naczk, M. (2004a). Antioxidant properties of food phenolics. In F. Shahidi, & M., Naczk (Ed.), *Phenolics in food and nutraceuticals* (pp. 403-437). Boca Raton, FL, USA: CRC Press.

Shahidi, F., & Naczk, M. (2004b). Nutritional and pharmacological effects of food phenolics. In F. Shahidi, & M., Naczk (Ed.), *Phenolics in food and nutraceuticals* (pp. 331–402). Boca Raton, FL, USA: CRC Press.

Shalaby, S. M., Zakora, M., & Otte, J. (2006). Performance of two commonly used angiotensin-converting enzyme inhibition assays using FA-PGG and HHL as substrates. *Journal of Dairy Research*, 73(2), 178-186.

Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H., & Hama, Y. (2008). Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *Journal of Applied Phycology*, 20(5), 705-711.

Shikama, K., & Matsuoka, A. (1986). Aplysia oxymyoglobin with an unusual stability property: kinetic analysis of the pH dependence. *Biochemistry*, 25(13), 3898-3903.

Shin, H. C., Hwang, H. J., Kang, K. J., & Lee, B. H. (2006). An antioxidative and antiinflammatory agent for potential treatment of osteoarthritis from *Ecklonia cava*. *Archives of pharmacal research*, 29(2), 165-171.

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *American Journal of Enology and Viticulture*, 16(3), 144-158.

Siriwardhana, N., Kim, K. N., Lee, K. W., Kim, S. H., Ha, J. H., Song, C. B. et al. (2008). Optimisation of hydrophilic antioxidant extraction from *Hizikia fusiformis* by integrating treatments of enzymes, heat and pH control. *International Journal of Food Science and Technology*, 43(4), 587-596.

Siriwardhana, N., Lee, K. W., Kim, S. H., Ha, J. W., & Jeon, Y. J. (2003). Antioxidant activity of *Hizikia fusiformis* on reactive oxygen species scavenging and lipid peroxidation inhibition. *Food Science and Technology International*, 9(5), 339-346.

Sivertsvik, M., Rosnes, J. T., & Kleiberg, G. H. (2003). Effect of modified atmosphere packaging and superchilled storage on the microbial and sensory quality of Atlantic salmon (*Salmo slar*) fillets. *Journal of Food Science*, 68(4), 1467-1472.

Sørensen, G., & Jørgensen, S. S. (1996). A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Z Lebensm Unter Forsch*, 202(3), 205-210.

Soyer, A., & Hultin, H. O. (2000). Kinetics of oxidation of the lipids and proteins of cod sarcoplasmic reticulum. *Journal of Agricultural and Food Chemistry*, 48(6), 2127-2134.

Stern, J. L., Hagerman, A. E., Steinberg, P. D., & Mason, P. K. (1996). Phlorotannin–protein interactions. *Journal of Chemical Ecology*, 22 (10), 1877-1899.

Stone, H., & Sidel, J. L. (1985). *Sensory evaluation practices* (pp. 311). Orlando, Fla.: Academic Press, Inc.

Su, L., Yin, J. J., Charles, D., Zhou, K. Q., Moore, J., & Yu, L. L. (2007). Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. *Food Chemistry*, 100(3), 990-997.

Tang, S., Sheehan, D., Buckley, D. J., Morrissey, P. A., & Kerry, J. P. (2001). Anti-oxidant activity of added tea catechins on lipid oxidation of raw minced red meat, poultry and fish muscle. *International Journal of Food Science and Technology*, 36(6), 685-692.

Targett, N. M., & Arnold, T. M. (1998). Minireview-Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *Journal of Phycology*, 34(2), 195-205.

Tichivangana, J. Z., & Morrissey, P. A. (1985). Metmyoglobin and inorganic metals as pro-oxidants in raw and cooked muscle systems, *Meat Science*, 15(2), 107-116.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2005). The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chemistry*, 93(4), 713-718.

Undeland, I. (2003a). Lipid oxidation in fish muscle–a water-soluble approach. http://www.lipidforum.org/news_online/nr65_may2003/oxidation.htm.

Undeland, I., Hultin, H. O., & Richards, M. P. (2002). Added triacylglycerols do not hasten hemoglobin-mediated lipid oxidation in washed minced cod muscle. *Journal of Agricultural and Food Chemistry*, 50(23), 6847-6853.

Undeland, I., Hultin, H. O., & Richards, M. P. (2003b). Aqueous extracts from some muscles inhibit hemoglobin-mediated oxidation of cod muscle membrane lipids. *Journal of Agricultural and Food Chemistry*, 51(10), 3111-3119.

Undeland, I., Kristinsson, H. G., & Hultin, H. O. (2004). Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. *Journal of Agricultural and Food Chemistry*, 52(14), 4444-4451.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, 39(1), 44-84.

Van Alstyne, K. L., McCarthy, J. J., Hustead, C. L., & Duggins, D. O. (1999). Geographic variation in polyphenolic levels of Northeastern Pacific kelps and rockweeds. *Marine Biology*, 133(2), 371-379.

Van Spreekens, K. J. A. (1974). The suitability of a modification of Long and Hammer's medium for the enumeration of more fastidious bacteria from fresh fishery products. *Archiv fur Lebensmittelhygiene*, 25(10), 213-219.

Vareltzis, P., & Hultin, H. O. (2007). Effect of low pH on the susceptibility of isolated cod (*Gadus morhua*) microsomes to lipid oxidation. *Journal of Agricultural and Food Chemistry*, 55(24), 9859-9867.

Vermeirssen, V., Van Camp, J., & Verstraete, W. (2002). Optimization and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *Journal of Biochemical and Biophysical Methods*, 51(1), 75-87.

Wagner, H., Elbl, G., Lotter, H., & Guinea, M. (1991). Evaluation of natural products as inhibitors of angiotensin I-converting enzyme (ACE). *Pharmaceutical and Pharmacological Letter*, 1, 15-18.

Waterman, P. G., & Mole, S. (1994). *Analysis of phenolic plant metabolites* (*Methods in ecology*) (pp. 238). Oxford, UK: Blackwell Scientific Publications.

Wettasinghe, M., & Shahidi, F. (2000). Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. *Food Chemistry,* 70(1), 17-26.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, 55(22), 8896-8907.

Xiong, Y. L., & Decker, E. A. (1995). Alterations of muscle protein functionality by oxidative and antioxidative processes. *Journal of Muscle Food*, 6(2), 139-160.

Yoshie, Y., Wang, W., Petillo, D., & Suzuki, T. (2000). Distribution of catechins in Japanese seaweeds. *Fisheries Science*, 66, 998-1000.

Yoshie-Stark, Y., Hsieh, Y. P., & Suzuki, T. (2003). Distribution of flavonoids and related compounds from seaweeds in Japan. *Journal of Tokyo University of Fisheries*, 89, 1-6.

Zaragozá, M. C., López, D., Sáiz, M. P., Poquet, M., Pérez, J., Puig-Parellada, P., et al. (2008). Toxicity and antioxidant activity in vitro and in vivo of two *Fucus vesiculosus* extracts. *Journal of Agricultural and Food Chemistry*, 56(17), 7773-7780.

PAPER I

I

Combined Application of Modified Atmosphere Packaging and Superchilled Storage to Extend the Shelf Life of Fresh Cod (*Gadus morhua*) Loins

Tao Wang; Kolbrún Sveinsdóttir; Hannes Magnússon; Emilía Martinsdóttir

Published in: J Food Sci. 73, 1 (2008) S11–S19

Combined Application of Modified Atmosphere Packaging and Superchilled Storage to Extend the Shelf Life of Fresh Cod (*Gadus morhua*) Loins

T. WANG, K. SVEINSDÓTTIR, H. MAGNÚSSON, AND E. MARTINSDÓTTIR

ABSTRACT: Development of new technologies and preservation methods to offer conveniently packed fish with sufficient keeping quality is important to meet increasing demand for value-added fresh fish products on the market. The aim of this study was to investigate the effect of combined application of modified atmosphere packaging (MAP) and superchilled storage on the shelf life of fresh cod loins. Fresh cod loins were packed in polystyrene boxes and in MA (CO_2/N_2/O_2: 50%/45%/5%) on day 3 postcatch and stored at chilled (1.5 °C) and superchilled (−0.9 °C) temperatures. Quantitative descriptive analysis (QDA) and physical, chemical, and microbial analyses were carried out during the 21 d of storage. Superchilled storage alone compared with traditional chilled storage in polystyrene boxes increased the total shelf life (days from catch) of cod loins from 9 to 16 or 17 d. Chilled MA packaging increased the shelf life from 9 to 14 d and when MAP and superchilled storage were combined, a synergistic effect was observed and the shelf life was further extended to at least 21 d. It is noteworthy that the characteristic fresh and sweet taste can be maintained longer under such conditions. This could contribute to enhanced eating quality of fresh cod fillets for consumers in distant markets. However, MAP combined with superchilled storage resulted in different textural properties. Superchilled MA packed cod loins had more meaty texture compared to other sample groups after 7-d storage.

Keywords: fresh cod loins, modified atmosphere packaging (MAP), quality changes, shelf life, superchilled storage

Introduction

The interest to extend the shelf life of fresh fish has promoted research on optimizing handling, refrigeration, and transport practices as well as packaging methods to maintain the high quality and safety of the products.

Modified atmosphere packaging (MAP) can significantly prolong the shelf life of a variety of seafoods at chill temperatures. Numerous studies have been carried out on the effect of MAP on the shelf life and quality retention of cod. The reported shelf life of MA packed cod ranges from 10 to about 20 d at 0 to 3 °C (Woyewoda and others 1984; Dalgaard and others 1993; Guldager and others 1998; Sivertsvik 2007), depending on initial microbiological quality, storage temperature, gas mixture, packaging materials, gas to product volume ratio, and so on (Church 1998; Sivertsvik and others 2002). However, some limitations of this packaging method were also reported. Modified atmosphere packaging usually extends the neutral taste period of fish rather than the initial fresh quality period (Shewan and others 1953). Too high CO_2 concentrations in the atmosphere may have negative impact on drip loss, color, texture, and flavor in the product. These can be explained by the pH drop caused by CO_2 dissolving in the muscle tissue, resulting in a decrease in the water-holding capacity of the proteins, the denaturation of muscle and pigment protein, as well as the development of sour odor and flavor (Huss 1995; Randell and others 1997; Masniyom and others 2002; Poli and others 2006).

Superchilling is one of the few promising techniques with the potential to preserve the prime quality of fresh fish. Different types of novel refrigeration systems have been widely used for the preservation of seafood products at subzero temperatures (−4 to 0 °C) such as slurry ice or ozone–slurry ice combined refrigeration system (Piñeiro and others 2004; Campos and others 2005, 2006), refrigerated seawater (Kraus 1992), subzero temperatures during storage (−2 and −3 °C) (Sivertsvik and others 2003), and the use of a cooling agent like dry ice (solid carbon dioxide) or a combination of dry ice and water ice (Jeyasekaran and others 2004, 2006). By using a new CBC (combined blast and contact) cooling technique followed by superchilled storage (−1.5 °C), the sensory shelf life of the cod fillets could be extended for at least 3 d compared to traditionally processed fillets stored at 0 °C, resulting in a shelf life of 15 d (Ólafsdóttir and others 2006b).

Modified atmosphere packaging has been shown to perform an effective synergy with superchilling in prolonging the shelf life of Atlantic mackerel (*Scomber scombrus* L.) fillets, smoked blue cod, and Atlantic salmon (*Salmo salar*) fillets (Penney and others 1994; Hong and others 1996; Sivertsvik and others 2003). The exact synergy mechanism is not yet fully elucidated but probably associated with increased dissolution of CO_2 at the superchilled temperature as well as the inhibition of specific spoilage organisms (SSOs) in the product (Sivertsvik and others 2003). Earlier studies in our laboratories showed that combined use of CBC cooling technique, MAP, and superchilled storage led to an increased sensory shelf life of 3 to 4 d compared to superchilled aerobic storage in expanded polystyrene (EPS) boxes (16 d) (Lauzon and Martinsdóttir 2005). However, there

MS 20070183 Submitted 3/12/2007, Accepted 9/18/2007. *Author Wang is with Dept. of Food Science and Engineering, Dalian Fisheries Univ., Heishijiao 52, Dalian, 116023, China. Author Wang is also with United Nations Univ., Fisheries Training Programme, Skulagata 4, 101 Reykjavik, Iceland. Authors Wang, Sveinsdóttir, Magnússon, and Martinsdóttir are with Matís ohf, Skúlagata 4, 101 Reykjavík, Iceland. Direct inquiries to author Wang (E-mail: wang_tao633@yahoo.com).*

doi: 10.1111/j.1750-3841.2007.00590.x

is little information available in the literature documenting the effect of combined application of modified atmosphere packaging and superchilled storage on the shelf life of fresh cod loins. Therefore, the objective of the present study was to determine the shelf life and quality changes of fresh cod loins under MAP and superchilled storage conditions, with an emphasis on the evolution pattern of sensory characteristics.

Material and Methods

Experimental design

The cod was caught with long line close to Sandgerði Southwest off Iceland in November 2005, bled, and stored in fish tubs containing flake ice. The average weight and length of the whole cod samples were 2.9 ± 0.5 kg and 69 ± 4 cm, respectively. The fish was gutted and headed the following day at Nyfiskur Co. (Iceland) and stored overnight in ice until further processing into loins 2-d postcatch. Fresh cod loin samples were taken directly from the processing line, and 5 kg cod loins (around 12 piece of loins) were placed in each of the insulated EPS boxes (160 × 400 × 263 mm) with false bottom, covered with plastic sheet and ice mats, and transported to the laboratory. The boxes were stored in a chilled storage chamber at 1 to 2 °C overnight before the storage trial started 3-d postcatch. A total of 260 fresh cod loins were randomly divided into 4 sample groups and kept under different storage conditions (Table 1). Three to 6 boxes of cod loins were directly kept at ambient temperature of 1.5 °C (group A1) and at −2.0 °C for the first 4 d followed by −0.9 °C for the remaining period (group A2). For sample groups M1 and M2, 1 to 2 cod loins (around 500 g) were packed in trays (EPS, Linstar E 39-34) with a built-in absorbent drip pad. Each tray was put into a vacuum bag (55PA/60LDPE, 25 × 40 cm, Samhentir VGI, Gardabaer, Iceland). Following removal of the air, a gas mixture (50% CO_2/45% N_2/5% O_2) was injected to modify the atmosphere. Group M1 was stored at 1.5 °C and group M2 at −2.0 °C for the first 4 d followed by −0.9 °C for the remainder.

During the storage trial, samples were taken from the 4 different groups at predetermined time intervals (Table 1). At each sampling time, 3 separate loins from groups A1 and A2 were homogenized aseptically and used first for microbiological analysis, then for TVB-N, TMA, and pH measurement. Four to 6 loins from groups A1 and A2 were used for sensory evaluation of cooked loins (QDA). For groups M1 and M2, 3 separate packs of cod loins from each group were used for microbiological, TVB-N, TMA, and pH measurements, and 2 separate packs from each batch were used for evaluation of drip loss and QDA. Gas composition of the modified atmosphere was determined for all 5 packs.

Temperature profile

Temperature data loggers (Stow Away®, Onset Computer Corp., Mass., U.S.A.) were used during all periods of chilled and superchilled storage to monitor the temperature of the ambient storage environment. One logger was placed at the top of boxes in each cold chamber to follow the environment variations of each storage condition. Temperature recordings were done at 3-min intervals.

Sensory evaluation

The quantitative descriptive analysis (QDA) method introduced by Stone and Sidel (1985) was used to evaluate the cooked cod loins. Nine to 12 panelists of the Icelandic Fisheries Laboratories' sensory panel participated in the QDA of the cooked samples. The members of the panel were previously trained in QDA for different cod products. Prior to the shelf life study, 1 session was held for training by using cod loin samples with different storage time in ice. The descriptors developed by Bonilla and others (2007) for fresh cod fillets and Magnússon and others (2006) for desalted cod fillets were used as a basis for this experiment during the training session. The list of descriptors was adapted to describe fresh and MA packed loins during the storage trial and the intensity of each attribute for a given sample was discussed under the guidance of the panel leader. The sensory attributes were evaluated using an unstructured scale (from 0% to 100%). Limit of sensory rejection was defined when the average QDA score for spoilage related attributes was above 20 as suggested by Magnússon and others (2006).

Samples weighing about 40 to 50 g were taken from the cod loins and each sample was placed in an aluminum box (8 × 5 × 4 cm) coded with 3-digit random numbers. The samples were cooked for 7 min in a steam oven preheated to at least 95 °C (Convotherm Elektrogeräte GmbH, Eglfing, Germany). A plastic lid was put on each box when removed from the oven and then served to the panelists.

All sample observations were conducted according to Intl. Standards (ISO 1988). The samples were evaluated randomly in duplicate in 1 to 2 sessions. A computerized sensory registration system (FIZZ Network, version 2.0, 1994–2000, Biosystèmes, Coutennon, France) was used for data recording and for further processing. Average scores of the judges were calculated for each sample from different storage groups and the reported values are the average of the duplicate samples.

Chemical measurements

Total volatile basic nitrogen (TVB-N) and trimethylamine (TMA) were determined in triplicate by the methods described by Malle and Poumeyrol (1989). The TVB-N measurement was performed by direct distillation into boric acid using a Kjeldahl-type distillatory (Struer TVN distillatory, STRUERS, Copenhagen, Denmark). The acid was back-titrated with diluted H_2SO_4 solution. To determine TMA, the same method was used as for TVB-N but adding 20 mL of 35% formaldehyde to the distillation flask to block the primary and secondary amines. The TVB-N and TMA content were expressed in mg N/100 g cod tissue. The pH was measured in triplicate by mixing 5 g of mince with 5 mL of deionized water using a Radiometer PHM80

Table 1 — Experimental design.

Treatment codes	Storage temperature (°C)	Packaging method	Sampling days[a]
A1	1.5 ± 0.2	Polystyrene boxes	0, 1, 4, 7
A2	−2.0 ± 0.3[b]	Polystyrene boxes	0, 1, 4, 7, 11, 13, 15, 18
	−0.9 ± 0.3[c]		
M1	1.5 ± 0.2	Plastic trays, MA packed	0, 1, 4, 7, 11, 13, 15,
M2	−2.0 ± 0.3[b]	Plastic trays, MA packed	0, 1, 4, 7, 11, 13, 15, 18, 21
	−0.9 ± 0.3[c]		

[a]Packing and 1st sampling (day 0) was on day 3 postcatch.
[b]From days 0 to 4.
[c]From days 4 to 21.

(Radiometer Analytical A/S, Copenhagen, Denmark). The pH meter was previously calibrated with buffer solutions of pH 7.00 ± 0.01 and 4.00 ± 0.01 at 20 °C.

Microbial analysis

Total viable psychrotrophic count (TVC) was determined by spread-plating aliquots on the surface of modified Long and Hammer's medium (LH) plates containing 1% NaCl (Van Spreekens 1974), incubated aerobically for 5 d at 15 °C. Selective counts of H_2S-producing bacteria were enumerated on iron agar (IA) plates as described by Gram and others (1987) with the exception that 1% NaCl was used instead of 0.5%. The plates were incubated at 15 °C for 5 d. Lactic acid bacterial (LAB) counts were evaluated by spread-plating aliquots on nitrite-actidione-polymyxin (NAP) (Davidson and Cronin 1973) plates and incubated at 22 °C for 5 d under microaerophilic conditions.

In all experiments, cooled maximum recovery diluent (MRD, Oxoid) was used for dilutions. The samples were analyzed in triplicate.

Gas and drip loss measurements

The gas composition in the headspace of all packs from MA storage was analyzed with a PBI Dansensor (Checkmate 9900, Denmark) by penetrating a needle through a gas-tight septum prior to opening the packs. The drip loss of the samples from groups M1 and M2 was measured in duplicate by gravimetric method after the samples had been kept for up to 1 h at refrigeration temperatures (2 to 4 °C). The mass of the drip (Δg) was divided by the initial mass of the product (g) and expressed as Δg/g (percent).

Statistical analysis

Analysis of variance (ANOVA) was carried out on all measured quality attributes in the statistical program NCSS 2000 (NCSS, Utah, U.S.A.). The program calculates multiple comparisons using Duncan's test to determine which sample groups are different. Significance of differences was defined at the 5% level ($P < 0.05$).

Principal component analysis (PCA) was conducted on sensory data using Unscrambler® (version 9.5, CAMO, Trondheim, Norway).

Results and Discussion

Ambient temperature profiles during storage

The average temperature of the cold chamber 1, in which groups A1 and M1 were stored, was 1.5 ± 0.2 °C. Cold storage chamber 2, in which groups A2 and M2 were stored, was −2.0 °C ± 0.3 °C from days 0 to 4 and −0.9 ± 0.3 °C after day 4 throughout the study period. Ice crystal formation was observed in cod loins stored under superchilled temperature at the early stage of storage at −2.0 °C.

Sensory evaluation

PCA was performed on QDA attributes to study the main tendencies in the data and to investigate the effect of different storage

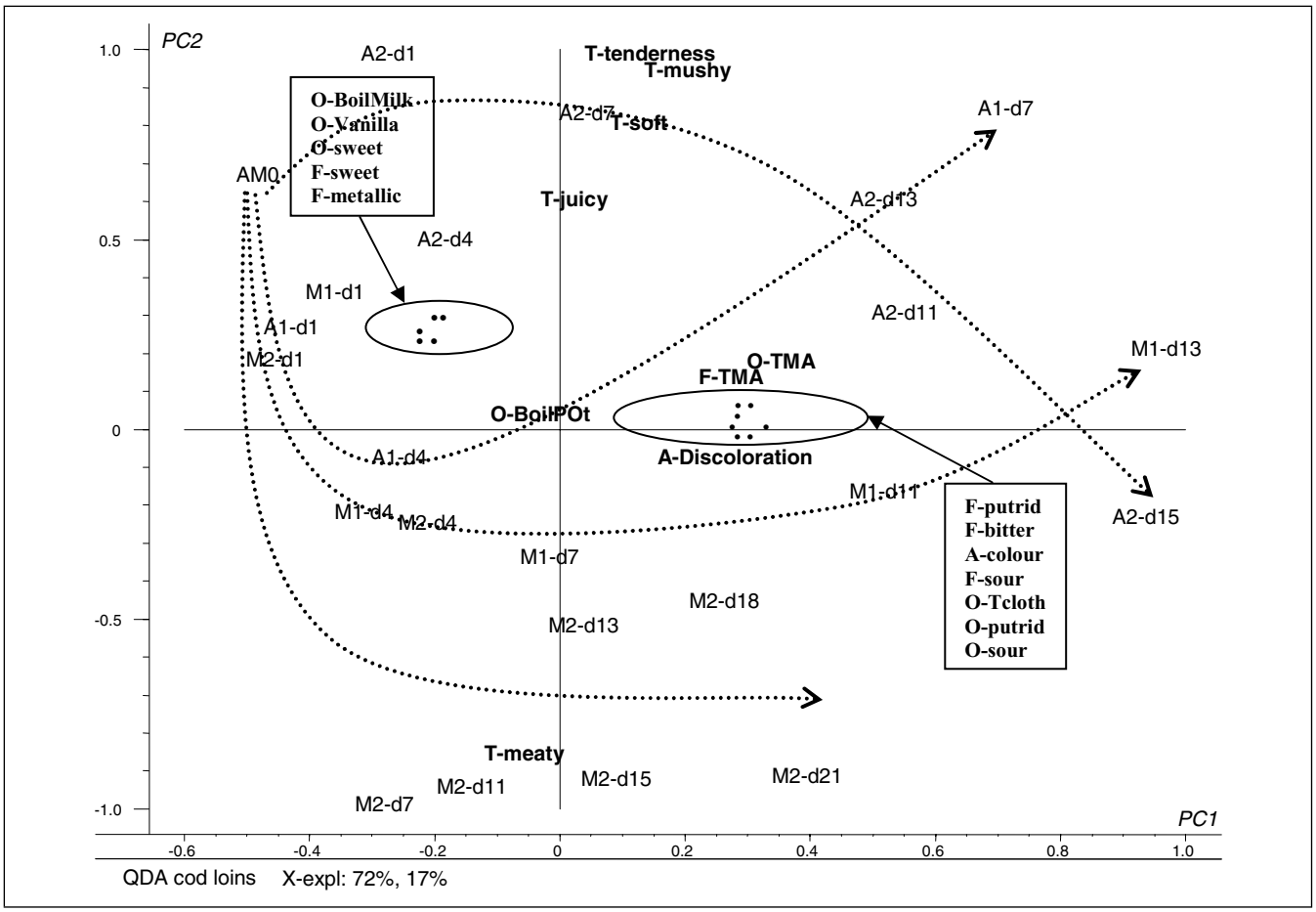


Figure 1 — PCA: Biplot of scores (samples) and loadings (sensory attributes). AM0-initial sample; F = flavor, O = odor, A = appearance, T = texture; dotted lines indicate evolution of each sample group with storage time.

conditions on the sensory quality of cod loins. Principal component 1 (PC1) and principal component 2 (PC2) accounted for 72% and 17% of the experimental variance between sample groups, respectively (Figure 1). The samples differed mainly with regard to odor and flavor attributes along PC1 which appeared to be substantially linked to the duration of storage. Fresh sample (AM0) located on the left side of the PCA plot received the highest scores for the attributes characteristic for cod at the beginning of storage time, such as sweet odor and flavor, metallic flavor, boiled milk, and vanilla odor. These characteristics became less evident as storage time progressed, but least change was observed in group M2 as seen by location of all M2 samples on the left side of the plot before

Table 2—Mean sensory scores of QDA attributes of cooked cod loins under different storage conditions.

A

		Odor								Appearance	
Storage period (d)	Group	Sweet	BoilMilk	BoilPot	Vanilla	Tcloth	TMA	Sour	Putrid	Color	Discolor
0	AM0	56	54	69	62	7	0	4	0	11	12
1	A1	57	46	36	29	4	0	2	0	13	15
	A2	54	50	37	31	5	0	3	0	19	23
	M1	54	42	33	28	4	0	3	0	19	19
	M2	54	43	39	28	6	0	2	0	11	16
	Significance										
4	A1	50	37	39	23	6	1	4	0	19	24
	A2	48	39	32	27	6	1	3	0	20	21
	M1	49	39	33	24	8	0	4	5	21	23
	M2	49	37	35	22	6	1	4	0	17	20
	Significance										
7	A1	21^{b}	20	23	8	32^{a}	38^{a}	26^{a}	17^{a}	34^{a}	43^{ab}
	A2	37^{a}	34	30	15	13^{b}	2^{b}	5^{b}	1^{b}	21^{b}	28^{cd}
	M1	34	33	32	15	19^{b}	4^{b}	11^{b}	4^{b}	26	37^{bc}
	M2	47^{a}	39	36	16	9^{b}	2^{b}	5^{b}	0^{b}	19^{b}	25^{d}
	Significance	***	*			***	***	***	***	**	***
11	A2	16^{b}	14^{b}	18^{b}	5^{b}	31^{a}	24^{a}	15	10	28	35^{a}
	M1	22^{b}	17^{b}	17^{b}	6^{b}	33^{a}	25^{a}	19^{a}	13^{a}	24	28
	M2	37^{a}	31^{a}	33^{a}	16^{a}	6^{b}	1^{b}	4^{b}	1^{b}	16	22^{b}
	Significance	*	*	*	**	***	***	*	*		
13	A2	24	21^{a}	22	9	24^{b}	24^{b}	14^{b}	10^{b}	26	33
	M1	11^{b}	9^{b}	11^{b}	4	40^{a}	61^{a}	30^{a}	25^{a}	29	40
	M2	35^{a}	23^{a}	32^{a}	11	7^{c}	3^{c}	6^{b}	4^{b}	25	31
	Significance	*	*	**		***	***	**	**		
15	A2	7^{b}	7^{b}	5^{b}	1^{b}	41	50^{a}	47^{a}	50^{a}	28	46
	M2	29^{a}	27^{a}	31^{a}	6^{a}	18	6^{b}	10^{b}	8^{b}	21	33
	Significance	**	**	***	*		***	***	***		

B

		Flavor						Texture				
Storage period (d)	Group	Sweet	Metallic	Sour	Bitter	TMA	Putrid	Soft	Juicy	Tender	Mushy	Meaty
0	AM0	61	65	7	7	0	0	61	71	79	32	43
1	A1	59	54	4	5	0	0^{b}	60	65	69	26^{b}	48^{a}
	A2	47	51	5	7	2	1^{a}	72	65	77	50^{a}	30^{b}
	M1	58	51	4	4	0	0	58	69	67	34^{b}	45^{a}
	M2	63	58	4	3	0	0^{b}	56	70	67	25^{b}	52^{a}
	Significance						*				**	*
4	A1	52	44	6	6	2	0	52	57	55^{b}	30	39
	A2	44	42	6	6	3	0	57	61	71^{a}	44	32
	M1	50	46	6	6	1	0	52	62	54^{b}	21	44
	M2	44	42	6	10	2	0	46	50	55^{b}	27	39
	Significance									*	*	
7	A1	22^{b}	15^{b}	25^{a}	22^{a}	31^{a}	18^{a}	77^{a}	57^{a}	77^{a}	59^{a}	14^{c}
	A2	43^{a}	29	7^{b}	9	3^{b}	1^{b}	74^{a}	58^{a}	75^{a}	56^{a}	18^{c}
	M1	43^{a}	35^{a}	8^{b}	9	3^{b}	1^{b}	57^{b}	53^{a}	58^{b}	25^{b}	48^{b}
	M2	51^{a}	45^{a}	5^{b}	10^{b}	1^{b}	0^{b}	39^{c}	43^{b}	40^{c}	14^{b}	61^{a}
	Significance	***	**	***		***	***	***	**	***	***	***
11	A2	23^{b}	15^{b}	11	17^{a}	17^{a}	12^{a}	65^{a}	54	70	52^{a}	21^{b}
	M1	21^{b}	17^{b}	15	18^{a}	24^{a}	13^{a}	57^{a}	51	61	38^{a}	32^{b}
	M2	39^{a}	32^{a}	6	5^{b}	2^{b}	1^{b}	42^{b}	45	46	20^{b}	61^{a}
	Significance	*			*	***	*	**		**	***	***
13	A2	20	12	13	16	22^{b}	11	71^{a}	56	72	58^{a}	19^{b}
	M1	10^{b}	6^{b}	18^{a}	19	38^{a}	16	63^{a}	53	63	51^{a}	17^{b}
	M2	34^{a}	26^{a}	6^{b}	7	4^{c}	5	51^{b}	51	53	28^{b}	44^{a}
	Significance	*	*	*		***		**			**	**
15	A2	$> 6^{b}$	12	37^{a}	26^{a}	33^{a}	40^{a}	58	52	61	34	21^{b}
	M2	31^{a}	29	$> 8^{b}$	$> 6^{b}$	2^{b}	1^{b}	47	44	42	21	47^{a}
	Significance	*		***	**	***	***			*		*

[a]AM0: Initial sample; groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C.
[b]Values in the same column followed by different superscript letters are significantly different (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

day 15. With extended storage, the samples were more described by boiled potato odor, then by bitter and sour flavor, discolored appearance, putrid flavor, putrid and sour odor, table-cloth odor, TMA flavor, and odor. These changes occurred over different time periods in the 4 sample groups and corresponded well with the process of quality deterioration evaluated by chemical and microbial analysis. Juicy, meaty, tenderness, and soft texture contributed little to PC1. On the other hand, PC2 primarily explained variation between samples with regard to texture parameters. Cod loins appeared to be juicier and more tender at the very beginning of storage. Tender, mushy, and soft texture described group A2 to a greater extent than other groups. Group M2 showed a trend toward meaty texture and M2 samples were all located on the lower part of the plot after 7 d. Group A1 showed some tendency to mushy and soft texture at the end of storage time.

Previous studies have shown that shelf life extension of fresh fish can be gained by MA packaging, but usually the period of moderate-to-low quality (neutral taste period) is prolonged rather than earlier stage of prime quality. At neutral phase, the fillets are described as being neutral in odor and flavor and have lost their distinct and pleasant fresh, sweet taste (Shewan and others 1953). The detailed evaluation of changes in sensory attributes shown herein indicated that the combination of MAP with superchilled storage is effective to extend the earlier stage when characteristic fresh QDA odor and flavor attributes are prominent (Table 2). This is in agreement with previous studies of Lauzon and Martinsdóttir (2005) indicating that characteristic "fresh fish" taste can be maintained longer by superchilling the fillets during processing in combination with MAP and superchilled storage. It also needs to be highlighted that group M2 received significantly higher scores for sweet, boiled milk and vanilla odor, sweet, and metallic flavor on days 11, 13, and 15 ($P < 0.05$) compared to groups A2 and M1 (Table 2). This is clearly demonstrated in Figure 2 and 3. The combination of MAP with superchilled storage resulted in a better maintenance of characteristic fresh odor and flavor after 11-d storage compared with MAP or superchilled storage alone. Accordingly, the development of unpleasant odor and flavor was effectively delayed under the superchilled MAP condition. In addition, superchilled MA packed cod loins received higher scores for meaty texture and lower scores for soft, tenderness, mushy texture on day 11 (Figure 4). This could be correlated to higher drip loss caused by cell destruction due to slow freezing during the first 4-d storage. These results strongly suggest that MAP in combination with superchilled storage is a very advantageous technology to effectively guarantee the high eating quality

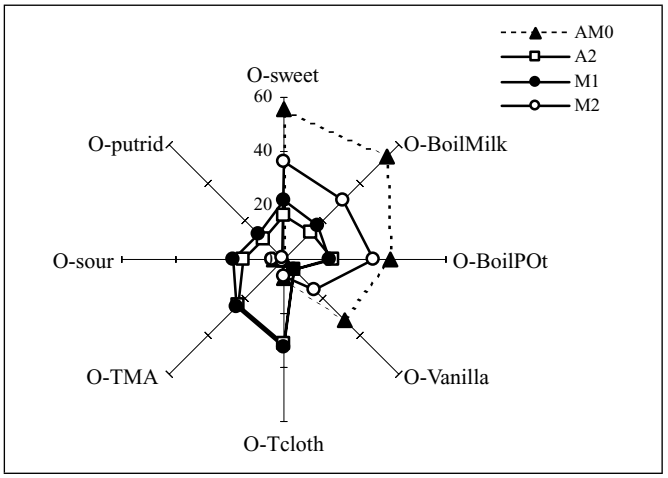


Figure 2—Radar plot of QDA odor attributes (mean scores) of different storage groups on day 11. AM0-initial sample; O = odor; groups M1 was stored at 1.5 °C; A2 and M2 at −1.0 °C.

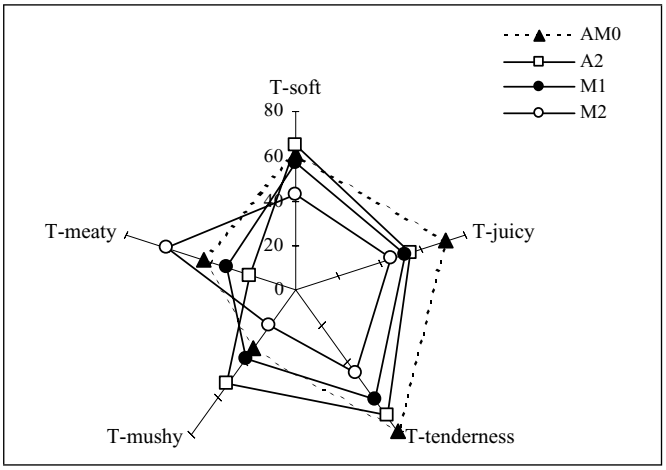


Figure 4—Radar plot of QDA texture attributes (mean scores) of different storage groups on day 11. AM0-initial sample; T = texture; groups M1 was stored at 1.5 °C; A2 and M2 at −1.0 °C.

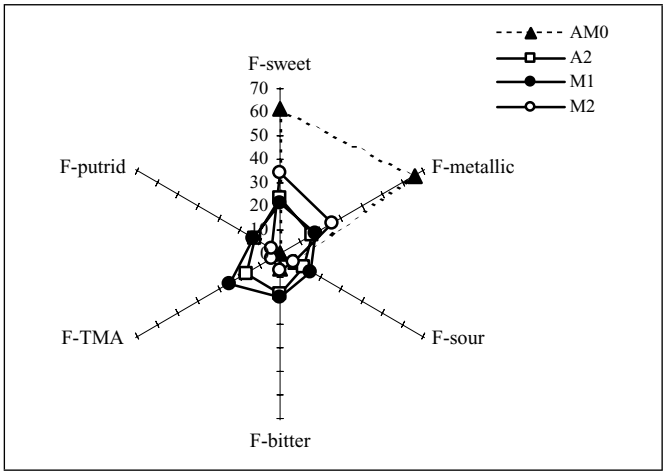


Figure 3—Radar plot of QDA flavor attributes (mean scores) of different storage groups on day 11. AM0-initial sample; F = flavor; groups M1 was stored at 1.5 °C; A2 and M2 at −1.0 °C.

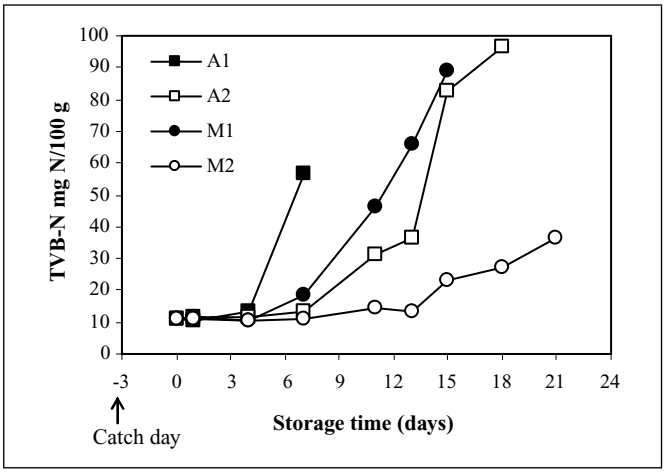


Figure 5—Total volatile basic nitrogen (TVB-N) formation of cod loins during storage. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

of fresh fish products, but careful temperature monitoring is also important to ensure the texture of superchilled products at a desirable level.

Chemical measurements

TVB-N values increased throughout the storage period for all groups (Figure 5). The initial lag phase for group A1 was 4 d and extended to 7 d for A2 and M1. After this time, TVB-N content increased rapidly. With regard to group M2, the production of TVB-N was effectively delayed and showed an almost horizontal profile up to day 13, indicating a significantly lower production of volatile bases under this condition. For TMA (Figure 6), the changing pattern was similar to that of TVB-N. High TVB-N production was generally correlated with high TMA production in groups A1, A2, and M1. TMA accounted for 70% to 80% TVB-N at sensory rejection time. The increase of pH values was most rapid in group A1. Lower pH values were recorded for the other groups (A2, M1, and M2) at the early stage of storage. A rapid increase of pH was observed from day 7 (M1) and 13 d (A2) onward. This is in agreement with TVB-N and TMA measurements. Interestingly, no marked increase in pH was noticed in group M2 except an increasing trend at the end of the storage period. Accordingly, in group M2 the pH value was always below 7 (Figure 7).

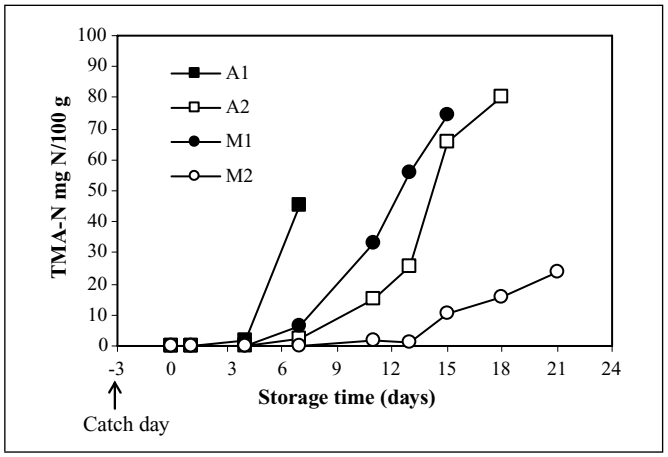


Figure 6—Trimethylamine (TMA) formation of cod loins during storage. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

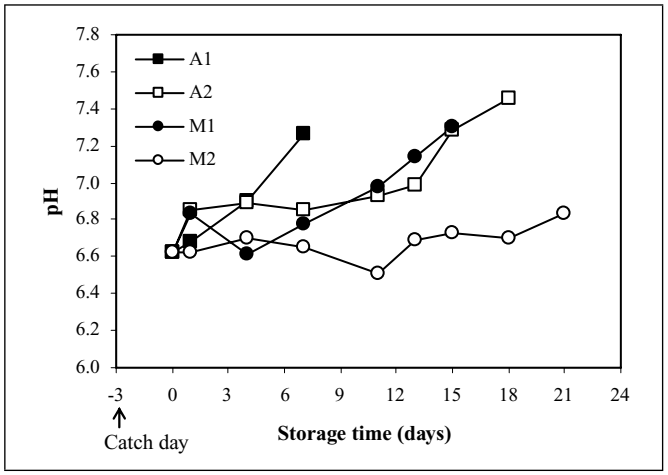


Figure 7—Changes in pH during storage of cod loins. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

Microbial analysis

The results from TVC on LH agar are shown in Figure 8. The microbial count of the fresh cod loins at the onset of the experiment was 5.3×10^4 colony forming units (CFU)/g. The microbial numbers increased most rapidly in the cod loins stored in polystyrene boxes at 1.5 °C (A1). MA packaging (M1) and superchilled storage (A2) effectively delayed the microbial growth. The lowest psychrotrophic bacterial count was obtained where both superchilling and MAP were used (M2) during the whole storage period ($P < 0.05$) and reached 2.6×10^7 CFU/g at the end of the storage trial (day 21). It needs to be noted that there was no marked difference between bacterial numbers on LH agar and those on IA when all colonies were counted in the latter medium (data not shown).

Changes in H_2S-producing bacteria of cod loins stored in 4 conditions are given in Figure 9. The growth rate of H_2S-producing bacteria under superchilled temperature (A2) was delayed only at the early stage of storage. After 4-d storage, a sharp increase was observed and their counts reached $>10^7$ CFU/g at sensory rejection on days 16 to 17. This is in agreement with previous studies on shelf life of superchilled cod fillets by Ólafsdóttir and others (2006b), who

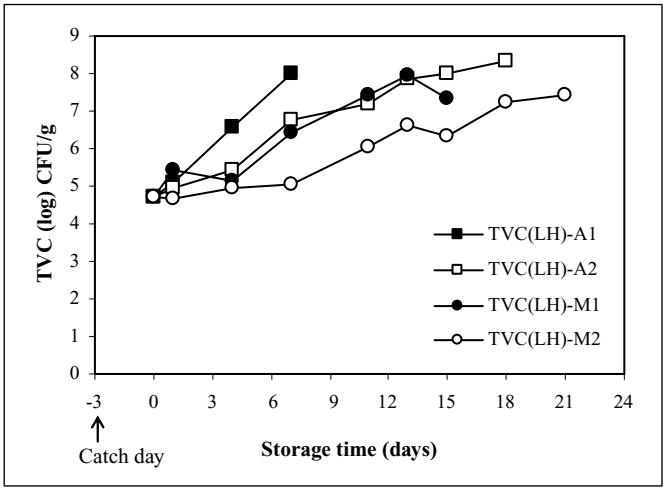


Figure 8—Total viable psychrotrophic bacteria counts (TVC) on LH at 15 °C in cod loins during storage. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

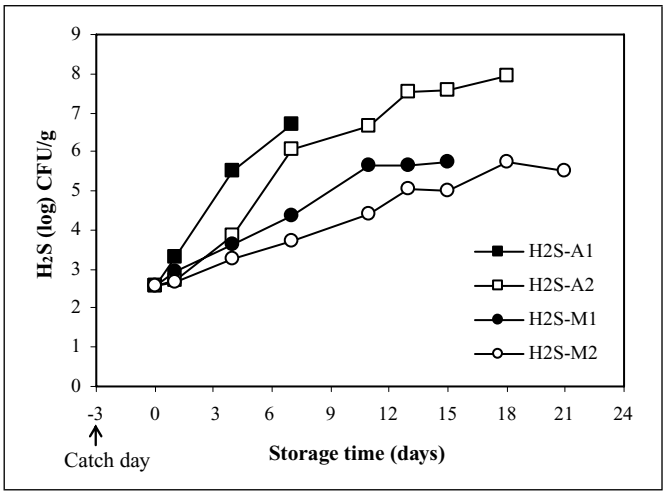


Figure 9—Growth of H_2S-producing bacteria on iron agar at 15 °C during storage of cod loins. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

also stated that H_2S-producing bacteria appeared to tolerate better the superchilled conditions than *Photobacterium phosphoreum* and pseudomonads. The effect of MA packaging alone (M1) was effective in slowing down the growth rate of H_2S-producing bacteria. H_2S-producing bacteria reached their maximum number after 11 d (4.7×10^5 CFU/g) and then remained fairly stable. Debevere and Boskou (1996) reported that MAP (60% CO_2, 40% O_2) had a strong inhibitory effect on the growth of H_2S-producing bacteria. When total aerobic plate counts reached log 6 CFU/g at rejection point, the number of H_2S-producing bacteria was only around log 3 CFU/g. The relatively higher counts in M1 at sensory rejection in our experiment might be attributed to the higher initial H_2S-producing bacteria load, lower CO_2 concentration (50% CO_2) used, and delayed application of MA packaging (3 d after catch). MAP combined with superchilled storage (M2) showed a great efficacy in controlling the growth of H_2S-producing bacteria compared with groups A2 and M1 ($P < 0.05$), reaching only around log 5.49 to 5.73 CFU/g at the later stages of storage.

Lower counts of H_2S-producers but high level of TMA (33 mg N/100 g) in group M1 at sensory rejection may indicate that other TMA producing bacteria like *P. phosphoreum* played an important role in the spoilage of chilled MAP cod loins as suggested earlier by other researchers (Dalgaard and others 1993, 1997; Dalgaard 1995).

Counts of presumptive lactic acid bacteria (LAB) on NAP agar are shown in Figure 10. From the 1st day on, an exponential increase was observed for groups A1, A2, and M1. Lactic acid bacteria (LAB) are carbon dioxide tolerant and CO_2 packing inhibits respiratory organisms and selects for LAB and facultative anaerobic bacterial species like *P. phosphoreum* (Debevere and Boskou 1996; Dalgaard 2000). Masniyom and others (2005) reported that LAB counts were

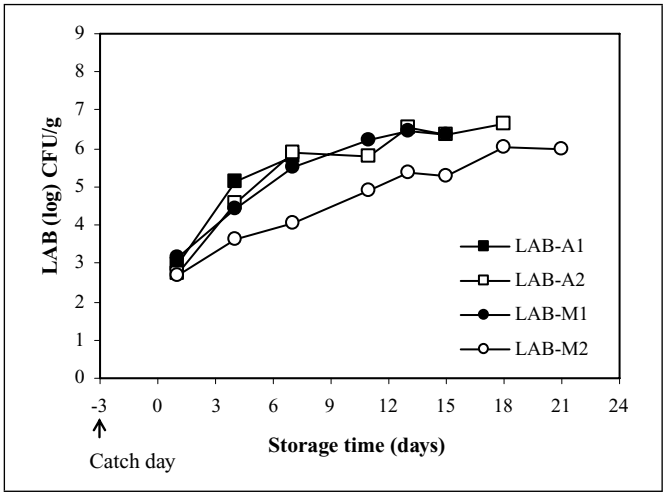


Figure 10—Growth of presumptive lactic acid bacteria (LAB) on NAP agar at 22 °C during storage of cod loins. Groups A1 and M1 were stored at 1.5 °C, A2 and M2 at −1.0 °C. Means of 3 samples.

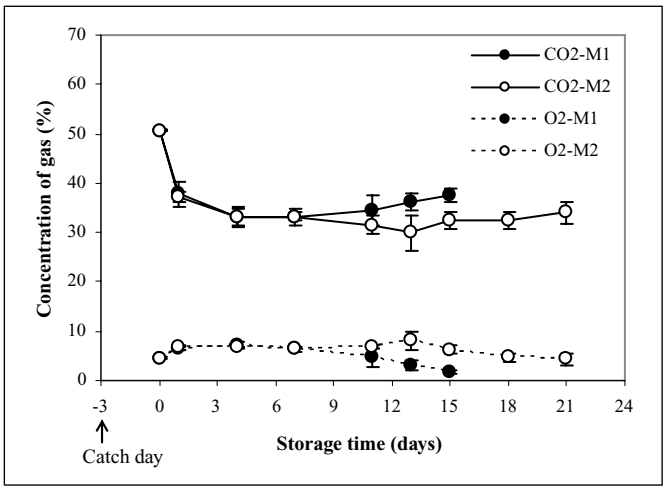


Figure 11—Change of headspace gas composition of the cod loins in MAP during storage. Group M1 was stored at 1.5 °C and M2 at −1.0 °C. Means of 2 samples.

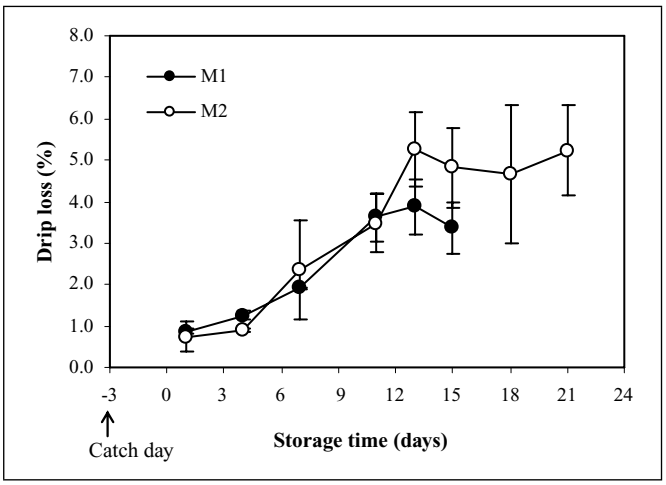


Figure 12—Changes in drip loss during storage of cod loins. Vertical bars represent SD. Group M1 was stored at 1.5 °C and M2 at −1.0 °C. Means of 2 samples.

Table 3—Overview of shelf life estimation and measured initial values for the microbial, TVB-N, TMA, pH, and final estimated values for the experimental data at sensory rejection for all experimental groups.

		Estimated values at sensory rejection			
	Initial values	A1	A2	M1	M2
Estimated shelf life (d)[a]		9	16 to 17	14	>24
Estimated shelf life (d)[b]		8	16	12	24
TVC (log_{10}CFU/g)	4.7	7.5	7.9 to 7.9	7.4	>7.4
H_2S-producer counts	2.6	6.7	7.5 to 7.5	5.7	>5.5
LAB counts	Nd	5.6	6.4 to 6.5	6.2	>6.0
TVB-N (mg N/100 g)	10.8	42.0	36.6 to 59.2	46.1	>36.3
TMA (mg N/100 g)	0.0	30.2	25.5 to 44.8	33.4	>24.0
pH	6.63	7.15	6.99 to 7.14	6.97	>6.84

[a]Total shelf life, including days from catch, based on the sensory evaluation of cooked loins (average QDA scores for attributes related to spoilage = 20).
[b]Total shelf life, including days from catch, based on TVB-N = 35 mg N/100 g.

generally higher in fresh sea bass slices kept under MAP compared with those stored in air. The reason for the high counts of LAB in the aerobically stored plastic covered cod loins in this study may be explained by the possibility that microaerophilic conditions developed in the polystyrene boxes, which favored the growth of LAB. A similar effect was suggested earlier regarding the predominating growth of *P. phosphoreum* in haddock fillets stored in Styrofoam boxes (Olafsdottir and others 2006a).

Even though superchilling and MAP alone had little effect in retarding the growth of LAB, a synergistic hindering effect was observed in superchilled MAP cod loins (M2).

Gas and drip loss measurements

Average initial gas composition immediately after packaging was $CO_2/O_2/N_2$: 50.7%/4.6%/44.7% (Figure 11). For both MAP groups, carbon dioxide concentration decreased rapidly at the beginning of the storage period due to gas dissolution into tissue liquids (Ruiz-Capillas and Moral 2001; Sivertsvik and others 2004), reaching 33.1% ± 1.8% 4 d after packaging (M1) and 29.9% ± 3.5% 13 d after packaging (M2). This is in agreement with the theory that solubility of CO_2 in water increases with decreasing temperature (Carroll and others 1991; Sivertsvik and others 2003). The content of CO_2 increased again due to bacterial and enzymatic activity after 7 d (M1) and 18 d (M2) of storage.

Drip loss from the samples increased with storage time for both MAP groups (Figure 12). No obvious differences were observed between groups M1 and M2 during the first 11 d of storage. Drip loss was higher in group M2 during the subsequent period of storage. However, no significant difference was found between these 2 groups. The drip loss was around 3.6% (day 11) in group M1, which was in accordance with the 4% to 8% reported level at the end of shelf life of fresh MAP cod ((Dalgaard and others 1993; Guldager and others 1998). Sivertsvik and others (2003) reported that superchilled MA storage did not lead to excessive drip. Chilled and superchilled MA salmon fillets had almost the same level of drip loss (around 3%). A possible reason for a relatively high drip loss in group M2 (5.2% at day 21) in the present study might be that partial freezing may have destroyed the muscle cells as a result of ice crystal growth during the first 4 d of storage at −2 °C.

Shelf life determination

End of shelf life is usually determined when sensory attributes related to spoilage such as TMA-like, sour, putrid odor and flavor become evident (Sveinsdottir and others 2002; Magnússon and others 2006; Bonilla and others 2007). Those odors and flavors have mainly microbial origin in traditionally stored chilled fish (Huss 1995) and can be explained by the accumulation of volatile degradation compounds (Olafsdottir 2005). When the average QDA score for spoilage related attributes was above 20 (on the scale 0 to 100), most of the panelists could detect those attributes, which indicated that the sample was approaching the end of shelf life (Magnússon and others 2006). Based on these criteria, the expected shelf life could be roughly calculated as 6 d for A1, 13 to 14 d for A2, and 11 d for M1, but M2 did not reach the end of shelf life within the time span of the experiment (21 d). Corresponding total shelf life from the catch for groups A1, A2, and M1 is 9 d, 16 to 17 d, and 14 d, respectively, and more than 24 d for M2 (Table 3). When using TVB-N value (35 mg N/100 g) as a criterion to determine the end of shelf life, a relatively shorter shelf life was estimated in agreement with earlier studies (Olafsdóttir and others 2006b). The difference between sensory and chemical data is around 1 d for groups A1 and A2 and more difference or 2 d for group M1 (Table 3).

Conclusions

Based primarily on sensory evaluation with QDA, but also on physical, chemical, and microbiological data, MAP in combination with superchilled storage is a very effective means to extend the shelf life of fresh cod loins. The promise of this new technique is the potential to maintain the intrinsic fresh and sweet taste of the cod loins longer. However, significant meaty texture was observed in superchilled MA-packed cod loins after 7-d storage, which indicates that strict temperature control is crucial to avoid a negative impact on the texture caused by ice crystal formation when the storage temperature is approaching the freezing point of the fish.

Acknowledgments

This study was carried out at the Icelandic Fisheries Laboratories (IFL) as a part of the Fisheries Training Program at the United Nations Univ. in 2005–2006. The study was financially supported by UNU-FTP and Integrated EU Project SEAFOODplus-*Project 2.2 SEAFOODSENSE*. The authors would like to thank the sensory panel at IFL and Dr. Tumi Tomasson, program director and Mr. Thor Asgeirsson, deputy program director for their valuable support and guidance. Special thanks are due to Hélène L. Lauzon and Páll Steinthórsson for their collaboration in microbial analysis and Dr. Gudrun Olafsdottir for valuable comments on the article.

References

Bonilla AC, Sveinsdóttir K, Martinsdóttir E. 2007. Development of quality index method (QIM) scheme for fresh cod (*Gadus morhua*) fillets and application in shelf life study. Food Control 18(4):352–8.

Campos CA, Rodríguez Ó, Losada V, Aubourg SP, Barros-Velázquez J. 2005. Effects of storage in ozonised slurry ice on the sensory and microbial quality of sardine (*Sardina pilchardus*). Int J Food Microbiol 103(2):121–30.

Campos CA, Losada V, Rodríguez Ó, Aubourg SP, Barros-Velázquez J. 2006. Evaluation of an ozone-slurry ice combined refrigeration system for the storage of farmed turbot (*Psetta maxima*). Food Chem 97(2):223–30.

Carroll JJ, Slupsky JD, Mather AE. 1991. The solubility of carbon dioxide in water at low pressure. J Phys Chem Ref Data 20:1201–9.

Church N. 1998. MAP fish and crustaceans—sensory enhancement. Food Sci Technol Today 12(2):73–83.

Dalgaard P. 1995. Qualitative and quantitative characterization of spoilage bacteria from packed fish. Int J Food Microbiol 26:319–33.

Dalgaard P. 2000. Fresh and lightly preserved seafood. In: Man CMD, Jones AA, editors. Shelf-life evaluation of foods. 2nd ed. Gaithersburg, Md.: Aspen Publishers Inc. p 110–39.

Dalgaard P, Gram L, Huss HH. 1993. Spoilage and shelf-life of cod fillets packed in vacuum or modified atmospheres. Int J Food Microbiol 19(4):283–94.

Dalgaard P, Mejlholm O, Christiansen TJ, Huss HH. 1997. Importance of *Photobacterium phosphoreum* in relation to spoilage of modified atmosphere-packed fish products. Lett Appl Microbiol 24(5):373–8.

Davidson AP, Cronin F. 1973. Medium for the selective enumeration of lactic acid bacteria from foods. Appl Microbiol 26(3):439–40.

Debevere J, Boskou G. 1996. Effect of modified atmosphere packaging on the TVB/TMA-producing microflora of cod fillet. Int J Food Microbiol 31:221–9.

Gram L, Trolle G, Huss HH. 1987. Detection of specific spoilage bacteria from fish stored at low (0 °C) and high (20 °C) temperatures. Int J Food Microbiol 4:65–72.

Guldager HS, Bøknæs N, Østerberg C, Nielsen J, Dalgaard P. 1998. Thawed cod fillets spoil less rapidly than unfrozen fillets when stored under modified atmosphere at 2 °C. J Food Prot 61(9):1129–36.

Hong LC, Leblanc EL, Hawrysh ZJ, Hardin RT. 1996. Quality of Atlantic mackerel (*Scomber scombrus* L.) fillets during modified atmosphere storage. J Food Sci 61(3):646–51.

Huss HH. 1995. Quality and quality changes in fresh fish. FAO Fisheries tech. paper T348. Rome: Food & Agriculture Organization of the United Nations & Danish Intl. Development Agency (FAO/DANIDA). 195 p.

ISO. 1988. Sensory analysis—general guidance for the design of test rooms. 8589. Genf, Switzerland: The Intl. Organization for Standardization. p 1–9.

Jeyasekaran G, Ganesan P, Jeya Shakila R, Maheswari K, Sukumar D. 2004. Dry ice as a novel chilling medium along with water ice for short-term preservation of fish Emperor breams, lethrinus (*Lethrinus miniatus*). Innov Food Sci Emerg Technol 5(4):485–93.

Jeyasekaran G, Ganesan P, Anandaraj R, Jeya Shakila R, Sukumar D. 2006. Quantitative and qualitative studies on the bacteriological quality of Indian white shrimp (*Penaeus indicus*) stored in dry ice. Food Microbiol 23(6):526–33.

Kraus L. 1992. Refrigerated sea water treatment of herring and mackerel for human consumption. In: Burt J, Hardy R, Whittle K, editors. Pelagic fish. The resource and its exploitation. Aberdeen, Scotland: Fishing News Books. p 73–81.

Lauzon HL, Martinsdóttir E. 2005. Combined use of CBC chilling, MAP and superchilling to extend the shelf life of cod fillets [abstract]. 35th WEFTA Annual Meeting; 2005 September 19–22; Antwerp, Belgium. Ijmuiden, the Netherlands: Netherlands Inst. for Fisheries Research (RIVO) and Oostende, Belgium: CLO-Gent, Sea Fisheries Dept.

Magnússon H, Sveinsdóttir K, Lauzon HL, Thorkelsdóttir Á, Martinsdóttir E. 2006. Keeping quality of desalted cod fillets in consumer packs. J Food Sci 71(2):M69–76.
Malle P, Poumeyrol M. 1989. A new chemical criterion for the quality control of fish: trimetylamine/total volatile basic nitrogen (%). J Food Prot 52:419–23.
Masniyom P, Benjakul S, Visessanguan W. 2002. Shelf-life extension of refrigerated sea bass slices under modified atmosphere packaging. J Sci Food Agric 82(8):873–80.
Masniyom P, Benjakul S, Visessanguan W. 2005. Combination effect of phosphate and modified atmosphere on quality and shelf-life extension of refrigerated sea bass slices. LWT—Food Sci Technol 38(7):745–56.
Ólafsdóttir G. 2005. Volatile compounds as quality indicators in fish during chilled storage: evaluation of microbial metabolites by an electronic nose [PhD thesis]. Faculty of Science, Univ. of Iceland, Reykjavik, Iceland. 291 p.
Olafsdottir G, Lauzon HL, Martinsdottir E, Kristbergsson K. 2006a. Influence of storage temperature on microbial spoilage characteristics of haddock fillets (*Melanogrammus aeglefinus*) evaluated by multivariate quality prediction. Int J Food Microbiol 111(2):112–25.
Olafsdottir G, Lauzon HL, Martinsdottir E, Oehlenschläger J, Kristbergsson K. 2006b. Evaluation of shelf-life of superchilled cod (*Gadus morhua*) fillets and the influence of temperature fluctuations during storage on microbial and chemical quality indicators. J Food Sci 71(2):S97–109.
Penney N, Bell RG, Cummings TL. 1994. Extension of the chilled storage life of smoked blue cod (*Parapercis colias*) by carbon dioxide packaging. Int J Food Sci Technol 29:167–78.
Piñeiro C, Barros-Velázquez J, Aubourg SP. 2004. Effects of newer slurry ice systems on the quality of aquatic food products: a comparative review versus flake ice chilling methods. Trends Food Sci Tech 15(12):575–82.
Poli BM, Messini A, Parisi G, Scappini F, Vigiani V, Giorgi G, Vincenzini M. 2006. Sensory, physical, chemical and microbiological changes in European sea bass (*Dicentrarchus labrax*) fillets packed under modified atmosphere/air or prepared from whole fish stored in ice. Int J Food Sci Technol 41(4):444–54.
Randell K, Hattula T, Ahvenainen R. 1997. Effect of packaging method on the quality of rainbow trout and Baltic herring fillets. Lebensm-Wiss Technol 30(1):56–61.
Ruiz-Capillas C, Moral A. 2001. Chilled bulk storage of gutted hake (*Merluccius merluccius* L.) in CO_2 and O_2 enriched controlled atmospheres. Food Chem 74(3):317–25.
Shewan JM, Macintosh RG, Tucker CG, Ehrenberg ASC. 1953. The development of a numerical scoring system for the sensory assessment of the spoilage of wet white fish stored in ice. J Sci Food Agric 4:283–98.
Sivertsvik M. 2007. The optimized modified atmosphere for packaging of pre-rigor filleted farmed cod (*Gadus morhua*) is 63 mL/100 mL oxygen and 37 mL/100 mL carbon dioxide. LWT—Food Sci Technol 40(3):430–8.
Sivertsvik M, Jeksrud WK, Rosnes JT. 2002. A review of modified atmosphere packaging of fish and fishery products—significance of microbial growth activities and safety. Int J Food Sci Technol 37(2):107–27.
Sivertsvik M, Rosnes JT, Kleiberg GH. 2003. Effect of modified atmosphere packaging and superchilled storage on the microbial and sensory quality of Atlantic salmon (*Salmo salar*) fillets. J Food Sci 68(4):1467–72.
Sivertsvik M, Rosnes JT, Jeksrud WK. 2004. Solubility and absorption rate of carbon dioxide into non-respiring foods. Part 2: raw fish fillets. J Food Eng 63(4):451–8.
Stone H, Sidel JL. 1985. Sensory evaluation practices. Orlando, Fla.: Academic Press Inc. 311 p.
Sveinsdottir K, Martinsdottir E, Hyldig G, Jorgensen B, Kristbergsson K. 2002. Application of quality index method (QIM) scheme in shelf-life study of farmed Atlantic salmon (*Salmo salar*). J Food Sci 67(4):1570–9.
Van Spreekens KJA. 1974. The suitability of Long and Hammers medium for the enumeration of more fastidious bacteria from fresh fishery products. Arch Lebensmittelhyg 25(10):213–9.
Woyewoda AD, Bligh EG, Shaw SJ. 1984. Controlled and modified atmosphere storage of cod fillets. Can Inst Food Sci Techno J 17(1):24–7.

PAPER II

II

Total Phenolic Compounds, Radical Scavenging and Metal Chelation of Extracts from Icelandic Seaweeds

Tao Wang; Rósa Jónsdóttir; Guðrún Ólafsdóttir

Published in: Food Chemistry. 116, 1 (2009) 240–248

Food Chemistry 116 (2009) 240–248

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds

Tao Wang [a,b,c,*], Rósa Jónsdóttir [b], Guðrún Ólafsdóttir [a,d]

[a] Department of Food Science and Nutrition, Faculty of Science, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland
[b] Matís ohf., Food Research, Innovation and Safety, Biotechnology and New Products, Skulagata 4, 101 Reykjavik, Iceland
[c] Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China
[d] Syni Laboratory Service, Lynghals 3, 110 Reykjavík, Iceland

ARTICLE INFO

Article history:
Received 28 May 2008
Received in revised form 2 February 2009
Accepted 17 February 2009

Keywords:
Icelandic seaweeds
Total phenolic contents (TPC)
Antioxidant activity
DPPH radical scavenging activity
ORAC
Ferrous ion-chelating ability
Multivariate data analysis

ABSTRACT

Screening of potential antioxidant activities of water and 70% acetone extracts from ten species of Icelandic seaweeds was performed using three antioxidant assays. Significant differences were observed both in total phenolic contents (TPC) and antioxidant activities of extracts from the various species evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, oxygen radical absorbance capacity (ORAC) and ferrous ion-chelating ability assays. Acetone extracts from three Fucoid species had the highest TPC and consequently exhibited the strongest radical scavenging activities. High correlation was found between TPC of seaweed extracts and their scavenging capacity against DPPH and peroxyl radicals, indicating an important role of algal polyphenols as chain-breaking antioxidants. However, water extracts generally had higher ferrous ion-chelating activity than 70% acetone extracts and no correlation was found with their TPC, suggesting that other components such as polysaccharides, proteins or peptides in the extracts were more effective chelators of ferrous ions than phenolic compounds.

1. Introduction

Natural antioxidants with multifunctional potential are of high interest as alternatives for synthetic antioxidants to prevent oxidation in complex food systems like muscle food. Numerous studies have focused on natural antioxidants in terrestrial plants and their application in food systems to prevent oxidation. Aquatic plants are also gaining interest as a potential source of antioxidants. Results have shown that marine macroalgae are a rich source of various natural antioxidants such as polyphenols, which play an important role in preventing lipid peroxidation. A series of polyphenolic compounds such as catechins (e.g. gallocatechin, epicatechin and catechin gallate), flavonols and flavonol glycosides have been identified from methanol extracts of red and brown algae (Santoso, Yoshie, & Suzuki, 2002; Yoshie, Wang, Petillo, & Suzuki, 2000; Yoshie-Stark, Hsieh, & Suzuki, 2003). Phlorotannins, a group of phenolic compounds which are restricted to polymers of phloroglucinol, have been identified from several brown algal families such as Alariaceae, Fucaceae and Sargassaceae. Many studies have shown that phlorotannins are the only phenolic group detected in brown algae (Jormalainen & Honkanen, 2004; Koivikko, Loponen, Pihlaja, & Jormalainen, 2007). Phlorotannins purified from several brown algae have been reported to possess strong antioxidant activity which may be associated with their unique molecular skeleton (Ahn et al., 2007). The multifunctional antioxidant activity of polyphenols is highly related to phenol rings which act as electron traps to scavenge peroxy, superoxide-anions and hydroxyl radicals. Phlorotannins from brown algae have up to eight interconnected rings. They are therefore more potent free radical scavenger than other polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings (Hemat, 2007). In addition, sulphated polysaccharides, carotenoid pigments including astaxanthin and fucoxanthin have also been demonstrated to possess excellent antioxidant potential (Kobayashi & Sakamoto, 1999; Miyashita & Hosokawa, 2008; Rupérez, Ahrazem, & Leal, 2002; Yan, Chuda, Suzuki, & Nagata, 1999; Zhao, Xue, & Li, 2008).

Because of the reported multifunctional properties of seaweed extracts, their exploitation as a source of natural antioxidants for application in complex food system like fish muscle is of interest. Lipid oxidation is a principal cause of quality deterioration in muscle foods during processing and storage, resulting in the production of rancid odours and unpleasant flavours, changes of colour and texture as well as lowering nutritional value of foods (Hultin, 1994; Jittrepotch, Ushio, & Ohshima, 2006). Lipid oxidation of

* Corresponding author. Address: Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China. Tel.: +86 411 84763557; fax: +86 411 84763508.
E-mail address: wang_tao633@yahoo.com (T. Wang).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.02.041

muscle phospholipids in fish may be induced by several catalysts, including iron from blood haemoglobin (Jónsdóttir, Bragadóttir, & Ólafsdóttir, 2007; Richards & Hultin, 2002). Many synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroxyquinone (TBHQ) and propyl gallate have been widely used in different food products. However, because of the potential health hazards, their use as food additives is under strict regulation in many countries. Moreover, BHT and α-tocopherol have been reported to be ineffective in retarding the oxidative deterioration in complex food system as fish muscle where both lipoxygenase (LOX) and haemeproteins are involved in the initiation of oxidation (He & Shahidi, 1997).

The first step in the search of a versatile antioxidant system based on seaweed is to characterise their antioxidant activity. When exploring the antioxidant potential of different seaweed species, the use of a single test is insufficient to identify the different mechanisms involved. Therefore, three antioxidant assays, DPPH radical scavenging activity, ferrous ion-chelating ability and ORAC, were chosen to evaluate the antioxidant activity of different seaweed species in this study. DPPH method measures the radical scavenging activity in organic systems and has been used extensively as a pre-screening method for new antioxidants from natural resources, due to its stability, simplicity, rapidity and reproducibility (Chen et al., 2008). Metal chelating capacity is claimed as one of the important mechanisms of antioxidant activity. The ferrous ions are the most powerful pro-oxidants among various species of transition metals present in food system (Hultin, 1994; Yomauchi, Tatsumi, Asano, Kato, & Ueno, 1988). However, oxidised haemeproteins such as met-haemoglobin and met-myoglobin have been reported to be more potent pro-oxidants than low-molecular-weight ferrous iron in fish muscle (Richards & Hultin, 2000; Undeland, Hultin, & Richards, 2003). In addition, ORAC assay was selected to measure the peroxyl radical absorption capacity of seaweed extracts. This methodology is regarded to be more biologically relevant than DPPH and other similar protocols and has been demonstrated to be especially useful for food samples and crude plant extracts when multiple constituents co-exist and complex reaction mechanisms are involved (Huang, Ou, & Prior, 2005). However, to our knowledge, no detailed studies have been performed on antioxidant potentials of seaweed extracts by using the ORAC assay.

The coastlines of Iceland are an abundant resource of seaweeds with broad species diversity, but little effort has been made to explore the antioxidant potential of seaweeds harvested in Iceland. Therefore, the aim of the present study was to screen for antioxidant activities in various types of edible Icelandic seaweeds by using three *in vitro* antioxidant activity assays and compare the effectiveness of water and solvent extraction. In addition, correlations between TPC and antioxidant activities were investigated to characterise the antioxidant properties. These pre-screening experiments reported herein will be a basis to selectively identify the most appropriate species for further characterisation and to evaluate suitability of active components from seaweed extracts as natural antioxidants for application in food muscle systems.

2. Materials and methods

2.1. Algal materials

Eight seaweed species, including six brown algae (Phaeophyta) (*Fucus vesiculosus* Linnaeus, *Fucus serratus* Linnaeus, *Laminaria hyperborea* (Gunnerus) Foslie, *Saccharina latissima* (Linnaeus) Lane, Mayes, Druehl and Saunders (= *Laminaria saccharina* (Linnaeus) Lamouroux), *Laminaria digitata* (Hudson) Lamouroux, *Alaria esculenta* (Linnaeus) Greville) and two red algae (Rhodophyta) (*Palmaria palmata* (Linnaeus) Kuntze, *Chondrus crispus* Stackhouse) were collected in Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland on March 19th, 2007. One brown algae (*Ascophyllum nodosum* (Linnaeus) LeJolis) and one green algae (Chlorophyta) (*Ulva lactuca* Linnaeus) were collected from the same area on May 16th, 2007. The freshly collected seaweeds were 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. For *L. hyperborea* and *S. latissima*, the stipes and hapteres were removed and the new and old parts of the blades were separated. The samples were lyophilised for 72 h, pulverised into powder and stored at −80 °C prior to extraction.

2.2. Chemicals

2,2́-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Iron (II) chloride and GC-grade acetone were purchased from Sigma–Aldrich (Steinheim, Germany). Fluorescein sodium salt (FL), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulphonic acid monosodium salt (Ferrozine), Folin–Ciocalteu's phenol reagent, phloroglucinol, α-tocopherol, citric acid (anhydrous) were obtained from Fluka (Buchs, Switzerland). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-methylphenol (BHT) and L-ascorbic acid were from Sigma–Aldrich (St. Louis, MO, USA). Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-$Na_2{\cdot}2H_2O$) was from ICN Biomedical Inc. (Aurora, OH, USA). All other reagents were of analytical grade.

2.3. Preparation of sample extract

Five grams of the algal powder was mixed with 100 ml of distilled water or 70% aqueous acetone (v/v), incubated in a platform shaker (Innova™ 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 3500 rpm for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. Acetone in the solvent extract was removed by rotary evaporation. The concentrate and the supernatant of water extract were freeze-dried and weighed. Each extraction was conducted in duplicate. The extraction yield was expressed as g dried extract/100 g dried algal powder. The dried duplicate extracts were pooled and stored at −80 °C until analysed. Each dried extract was then re-dissolved in distilled water at a concentration of 5 mg/ml as a stock solution. The stock solution was used both for the determination of TPC and antioxidant activities.

2.4. Determination of total phenolic content

The TPC of seaweed extract was determined in accordance with a protocol described by Turkmen, Sari, and Velioglu (2005) with minor modifications. One millilitre aliquot of each diluted sample (the extract stock solutions further diluted with distilled water, concentration ranged from 0.25 (*F. vesiculosus*) to 5 mg/ml (*L. digitata*)) was mixed with 5 ml of Folin–Ciocalteu reagent (10% in distilled water) in a test tube. After 5 min, 4 ml of sodium carbonate (7.5% in distilled water) were added to each tube, the test tubes were cap-screwed and vortexed. The samples were incubated for 2 h at room temperature in the darkness. The absorbance was measured at 725 nm with a UV–vis spectrophotometer (Ultrospec 3000 pro, Amersham Pharmacia Biotech, Ltd., Cambridge, UK). A standard curve with serial phloroglucinol solutions (ranging from 20 μg/ml to 100 μg/ml) was used for calibration. The analyses were done in triplicate. Results were expressed as gram of phloroglucinol equivalents (PGE) per 100 g of extract.

2.5. DPPH radical scavenging activity assay

The method of Brand-Williams, Cuvelier, and Berset (1995) as modified by Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) was used for measuring the DPPH radical scavenging ability of seaweed extracts.

The extract stock solutions were further diluted with distilled water at various ratios (at least 5 different dilutions were prepared for each extract) based on their free radical scavenging activities. An aliquot of each dilution (0.1 ml) was added to 3.9 ml of DPPH· (64 μM or 2.5×10^{-2} g/l in methanol prepared daily). The mixtures were allowed to stand for 2 h at room temperature. The absorbance was measured at 515 nm with the Ultrospec 3000 pro UV–vis spectrophotometer. L-ascorbic acid, BHT and α-tocopherol were used as reference standards. All measurements were performed in duplicate.

The calibration curve made with DPPH· between 4.15×10^{-3} and 4.15×10^{-2} g/l was used to calculate the remaining concentration of DPPH· in the reaction medium.

The percentage of remaining DPPH· (%$DPPH^{\cdot}_R$) was calculated as follows:

$$\%[DPPH^{\cdot}_R] = [(DPPH^{\cdot})_T/(DPPH^{\cdot})_{T=0}] \times 100$$

where $(DPPH^{\cdot})_{T=0}$ was the concentration of DPPH· at time zero (initial concentration) and $(DPPH^{\cdot})_T$ was the concentration of DPPH· after 2 h.

The percentage of remaining DPPH· was plotted against the sample/standard concentration to obtain EC_{50} value, which represents the concentration of the extract or standard antioxidant (mg/ml) required to scavenge 50% of the DPPH· in the reaction mixture. Its reciprocal, the antiradical power (ARP, ARP = $1/EC_{50}$) was also calculated for each of the extracts.

2.6. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to Davalos, Gomez-Cordoves, and Bartolome (2004) with slight modifications. Mx300 real-time PCR System (Stratagene Inc., La Jolla, CA) was used for the fluorescence measurements controlled by MxPro computer program. All reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). The extract stock solutions were further diluted appropriately with 75 mM, pH 7.4 sodium phosphate buffer (concentration range from 0.005 (*F. vesiculosus*) to 1 mg/ml (*L. digitata*)). Ten microlitres of each diluted sample were placed in the well (200 μl) of a microplate. Afterwards 60 μl fluorescein (70 nM) were added and the mixtures were pre-incubated at 37 °C for 15 min. Subsequently, 30 μl AAPH (12 mM) were added rapidly using a multichannel pipette to initiate the oxidation reaction. The microplate was placed immediately in the reader and the fluorescence (FAM) was recorded every 0.1 min for the first 20 cycles and every minute thereafter until the fluorescence of the last reading declined to <5% of the initial value (around 80 min). Excitation and emission filter wavelengths were set at 484 nm and 520 nm, respectively. 75 mM phosphate buffer (pH 7.4) was used as a blank. At least three independent assays were performed for each sample.

The antioxidant curves (fluorescence versus time) were normalised. The data from the curves were multiplied by the factor:

$$\frac{\text{fluorescence}_{\text{blank},t=0}}{\text{fluorescence}_{\text{sample},t=0}}$$

The area under the fluorescence decay curve (AUC) was calculated by the normalised curves with the following equation:

$$AUC = (0.5 + f_1/f_0 + f_2/f_0 + \cdots + f_{20}/f_0) \times 0.1 + (f_{21}/f_0 + f_{22}/f_0 + \cdots + f_{79}/f_0) \times 1 + 0.5 \times (f_{80}/f_0)$$

where f_0 was the fluorescence reading at the initiation of the reaction and f_{80} was the last measurement.

The net AUC was obtained by subtracting the AUC of the blank from that of a sample or standard. The ORAC value was calculated and expressed as micromoles of Trolox equivalents per gram extract (μmol of TE/g extract) using the calibration curve of Trolox.

2.7. Ferrous ion-chelating ability assay

The ferrous ion-chelating ability was determined according to the method of Decker and Welch (1990) with minor modifications. One hundred microlitres of each extract stock solution (5 mg/ml) were mixed with 135 μl of distilled water and 5 μl of 2 mM $FeCl_2$ in a microplate. The reaction was initiated by the addition of 10 μl of 5 mM ferrozine. The solutions were well mixed and allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm with a Tecan Sunrise microplate reader (Tecan Austria Gesellschaft, Salzburg, Austria). Distilled water (100 μl) instead of sample solution was used as a control. Distilled water (10 μl) instead of ferrozine solution was used as a blank, which is used for error correction because of unequal colour of the sample solutions. L-ascorbic acid, citric acid and EDTA-Na_2 were used as reference standards. All measurements were performed in triplicate. The ferrous ion-chelating ability was calculated as follows:

$$\text{Ferrous ion} - \text{chelating ability}(\%) = [(A_0 - (A_1 - A_2)]/A_0 \times 100.$$

where A_0 was the absorbance of the control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.8. Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's test was carried out to test for differences between species and extractants (water and 70% acetone) in the statistical program NCSS 2000 (NCSS, Kaysville, Utah, USA). Significance of differences was defined at the 5% level ($p < 0.05$). Multivariate analysis was performed by the Unscrambler 9.7 software package (CAMO AS, Trondheim, Norway). The main variance in the data set was detected using principal component analysis (PCA). All data were mean centred and scaled to equal unit variance prior to PCA. Full cross-validation was used in the validation models. Partial least square regression (PLSR) was used to test the quantitative correlation between TPC and antioxidant properties of all the water and 70% acetone extracts. DPPH, ORAC and chelating activity were used as *X* predictors and TPC as *Y* response factor.

3. Results and discussion

3.1. Extraction yield

Considerable variations in extraction yield were found among different seaweed species (data not shown). The highest extraction yield was recorded for the water extract of *U. lactuca* (44.7 g of dried extract/100 g dried algal powder) whereas the lowest for 70% acetone extract of *C. crispus* (10.5 g of dried extract/100 g dried algal powder). Extractants also have an impact on the yield. For most seaweed species, the extraction yields of water extracts were higher than those of 70% acetone extracts which indicated that most of the soluble components in seaweeds were high in polarity. It should also be noted that the water extracts of *L. hyperborea* (both old and new blades) and *S. latissima* (new blades) were very viscous and difficult to filtrate through the filter paper due to the high content of alginate in the extracts, resulting in low extraction yield. Interestingly, large differences were observed between water

extracts and 70% acetone extracts for some species such as *U. lactuca*, *P. palmata* and *C. crispus*. These seaweeds are known to contain high levels of water-soluble components, such as soluble polysaccharides, protein and peptides (Galland-Irmouli et al., 1999) which were poorly extracted by 70% acetone.

3.2. Total phenolic content

Significant differences were found in TPC among different seaweed species, ranging from 0.4 to 24.2 g PGE/100 g extract (Fig. 1). Brown algae generally contained higher amounts of polyphenols than red and green algae. Intra-thallus variation in TPC was also observed both in *L. hyperborea* and *S. latissima*. Old blades had higher TPC values compared to new blades. This is in agreement with earlier studies by Connan, Delisle, Deslandes, and Ar Gall (2006) who reported that there was a high correlation between the age of the tissues and TPC for *L. hyperborea*. High levels of TPC were found in fucoid seaweed species. The TPC in 70% acetone extracts of *A. nodosum*, *F. serratus* and *F. vesiculosus* were 15.9, 24.0 and 24.2 g PGE/ 100 g extract, respectively while the TPC of the water extracts of *A. nodosum*, *F. serratus* and *F. vesiculosus* were 13.8, 16.9 and 17.6 g PGE/100 g extract, respectively. The highest amount of TPC was observed for 70% acetone extract of *F. vesiculosus*. Similar levels of TPC were reported for 70% acetone extract (30 g PGE /100 g extract on dry weight basis) and methanol extract (23.21 g PGE /100 g extract) of *F. vesiculosus* from the Finnish Archipelago Sea and Atlantic coast of Canada (Jormalainen, Honkanen, Vesakoski, & Koivikko, 2005; Zhang et al., 2006).

For most seaweed species, 70% aqueous acetone was more efficient to extract polyphenolic compounds compared to water (Fig. 1). Phenolic compounds are generally more soluble in polar organic solvents than in water. The effective extractants recommended are aqueous mixtures of methanol, ethanol and acetone (Waterman & Mole, 1994). Koivikko, Loponen, Honkanen, and Jormalainen (2005) compared the extraction efficacy of soluble polyphenols (mostly phlorotannins) from *F. vesiculosus* by using eight extractants with different polarities. 70% aqueous acetone (v/v) was found to be the most efficient solvent. It has been postulated that acetone has the ability to inhibit protein–polyphenol complex formation during extraction (Hagerman, 1988) or even break down hydrogen bonds formed between phenolic group and protein carboxyl group (Kallithraka, Garcia-Viguera, Bridle, & Bakker, 1995). On the contrary, other compounds such as water-soluble polysaccharides, proteins and organic acids were simultaneously extracted when using water as only extractant (Chirinos, Rogez, Campos, Pedreschi, & Larondelle, 2007). Because of safety concerns regarding the use of some organic solvent extracts in food, the optimization of extraction of polyphenols from *F. vesiculosus* by using approved food-grade solvent such as food-grade alcohol and ethyl acetate is now in progress in our laboratory.

3.3. Antioxidant activity

3.3.1. DPPH radical scavenging activity

The DPPH radical scavenging activity of water and 70% acetone seaweed extracts increased in a concentration-dependent manner and also increased with the increment of the incubation time (data not shown).

The comparison of DPPH radical scavenging activity of water and 70% aqueous acetone extracts of seaweeds are shown in Fig. 2. Generally, 70% aqueous acetone extracted more compounds with scavenging abilities on DPPH radicals than water. As pointed out by some researchers, change in extractant polarity alters its efficacy to extract a specific group of antioxidant compounds and influences the antioxidant properties of the extracts. (Zhou & Yu, 2004). Among all of the 70% acetone extracts, *F. vesiculosus* exhibited the most effective scavenging ability on DPPH radicals (ARP = 93.9, $EC_{50} = 10.7\times10^{-3}$ mg/ml), followed by *F. serratus* (ARP = 90.8, $EC_{50} = 11.0\times10^{-3}$ mg/ml), *A. nodosum* (ARP = 54.1, $EC_{50} = 18.5\times10^{-3}$ mg/ml), the old blades of *L. hyperborea* (ARP = 38.8, $EC_{50} = 25.8\times10^{-3}$ mg/ml) and *S. latissima* (ARP = 33.6, $EC_{50} = 29.8\times10^{-3}$ mg/ml). The other extracts showed relatively weak scavenging potentials. The lowest ARP value (0.4, EC_{50} = 2.5 mg/ml) was found in 70% acetone extract of *C. crispus*, which was approximately 230-fold lower than that of *F. vesiculosus* (Fig. 2). It was observed that the extracts containing high levels of TPC were also potent DPPH radical scavenger, suggesting that algal polyphenols may be the principal constituents responsible for the antiradical properties of the extracts. Nevertheless, it should be pointed out that because of the non-specifity of 70% acetone extraction, other classes of antioxidant compounds such as fucoxanthin and sterols could be partially and simultaneously extracted and thus may have contributed to the overall activities.

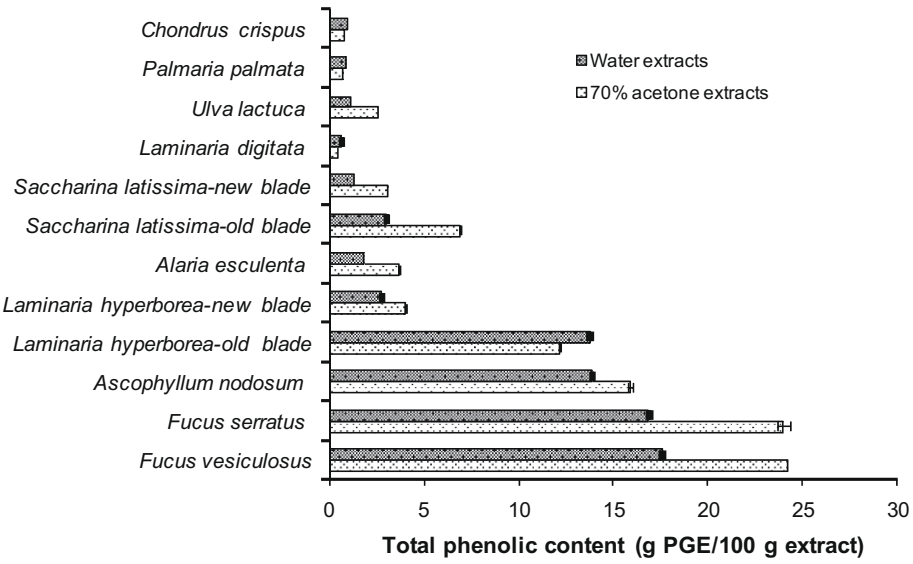


Fig. 1. Total phenolic content (TPC) in water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds. Values are means ± S.D. (n = 3).

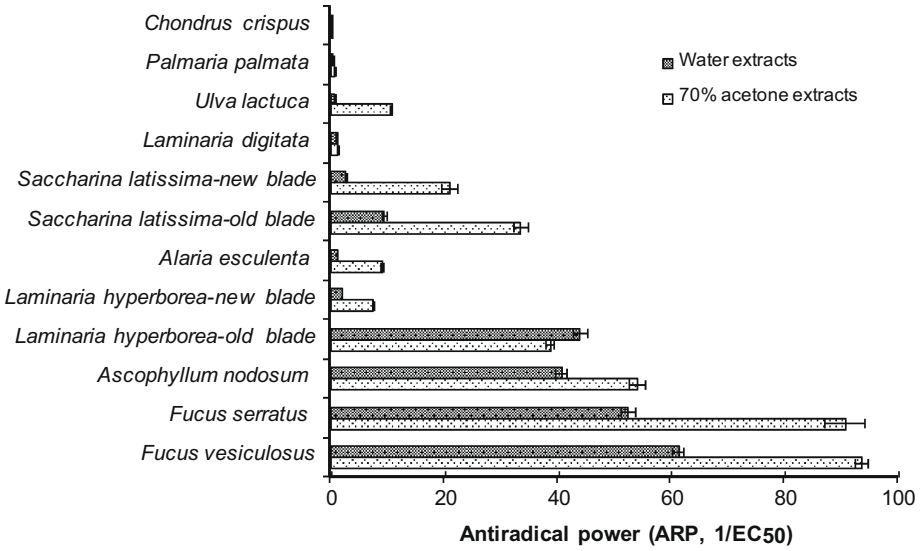


Fig. 2. DPPH radical scavenging activity (antiradical power (ARP); ARP = $1/EC_{50}$) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds ($n = 2$, bars are mean dev.).

All the reference compounds, ascorbic acid (ARP = 400.9, $EC_{50} = 2.5\times10^{-3}$ mg/ml), BHT (ARP = 305.2, $EC_{50} = 3.3\times10^{-3}$ mg/ml) and α-tocopherol (ARP = 168.6, $EC_{50} = 5.9\times10^{-3}$ mg/ml), exhibited higher DPPH radical scavenging effect when compared with all the seaweed extracts tested.

Because of different extraction, measurement methods and units used in the various antioxidant activity studies on seaweed reported in the literature, direct comparison of our results on radical scavenging activity of seaweed extracts with other studies is not feasible. However, similar tendency was observed by Jiménez-Escrig, Jiménez-Jiménez, Pulido, and Saura-Calixto (2001) who reported that brown seaweeds generally showed better DPPH scavenging capacity than red seaweeds. The highest scavenging activity was recorded in *Fucus* species and no activity was detected for *C. crispus*.

3.3.2. ORAC

Further evaluation of the chain-breaking antioxidant activities of different Icelandic seaweed extracts was conducted by ORAC-FL assay. The peroxyl radical scavenging activities of seaweed extracts varied considerably from 4 to 2567 μmol of TE/ g extract, depending on seaweed species and extractant used (Fig. 3). In general, 70% aqueous acetone extracts showed higher scavenging activities against peroxyl radicals than water extracts. The highest ORAC value was determined in the 70% acetone extract from *F. vesiculosus* (2567 μmol TE/g extract), followed by *F. serratus* (2545 μmol TE/g extract), *A. nodosum* (1417 μmol TE/g extract) and old blades of *L. hyperborea* (975 μmol TE/g extract). *L. digitata* showed very low peroxyl radical scavenging activity, which was around 640 times weaker than that of *F. vesiculosus*.

Because of lack of ORAC data on seaweeds, our results cannot be evaluated by comparing with peroxyl radical scavenging activities of other seaweed species. However, the ORAC values of three fucoid species in the present study were comparable to or even higher than those reported in the literature for various high antioxidant fruit and spice extracts, including 50% acetone extract of strawberry (540 μmol of TE/g extract) (Huang, Ou, Woodill, Flanagan, & Prior, 2002), 80% ethanol extract of blackberry (674.2 μmol of TE/g extract), (Elisia, Hu, Popovich, & Kitts, 2007), 50% acetone extract of cinnamon (1256 μmol of TE/g extract), (Su et al., 2007), but still lower than methanol extract of rosemary (2800–4360 μmol of TE/g extract) (Ho, Tsai, Tsai, & Lin, 2008; Tsai, Tsai, Chien, Lee, & Tsai, 2008) and Nutmeg (3570 μmol of TE/g extract) (Ho et al., 2008).

3.3.3. Ferrous ion-chelating activity

Metal chelating activities of all algal extracts were tested at a concentration of 5 mg/ml. In general, water extracts showed significantly higher ferrous ion-chelating capacity than 70% acetone extracts (Fig. 4). In accordance with DPPH and ORAC results, potent chelation abilities were again detected in the water extracts from three fucoid species, near or above 95% at 5 mg/ml. Interestingly, however, water extracts from *P. palmata* and *C. crispus* with significantly lower polyphenol levels as well as weaker scavenging activities against DPPH and peroxyl radicals, also exhibited equivalent chelating effects. The high binding capacities to different heavy metals of algal dietary fibres such as alginate, fucoidan from brown algae and carrageenan, agar from red algae are well documented. However, there are contradictory reports in the literature regarding metal chelating capacities of polyphenols. Some studies have demonstrated that polyphenols derived from brown seaweeds are potent ferrous ion chelators (Chew, Lim, Omar, & Khoo, 2008; Senevirathne et al., 2006) and metal chelating potency of phenolic compounds are dependent upon their unique phenolic structure and the number and location of the hydroxyl groups (Santoso, Yoshie-Stark, & Suzuki, 2004). In contrast, other authors have claimed that metal chelation play a minor role in the overall antioxidant activities of some polyphenols (Rice-Evans, Miller, & Paganga, 1996). The study conducted by Toth and Pavia (2000) showed that other compounds such as polysaccharides (e.g. alginates and fucoidan) and/or phytochelatins were more effective than phlorotannins for the detoxification and resistance to copper accumulation in *A. nodosum*. Andjelkovic et al. (2006) reported that the ability of phenolic compounds to chelate iron were far lower than that of EDTA. In addition, some peptides as well as proteins have also been reported to possess the abilities to chelate metal ions (Saiga, Tanabe, & Nishimura, 2003).

For comparison, several standards were also tested in this experiment. EDTA-Na_2 was excellent chelator for ferrous ions and its chelating capacity was 99.8% at a concentration as low as 0.05 mg/ml, much higher than all the seaweed extracts. Citric acid showed considerably lower effect of 21.5% at 5 mg/ml. Ascorbic acid had rather weak chelating capacity, which was only 8.2% at 20 mg/ml.

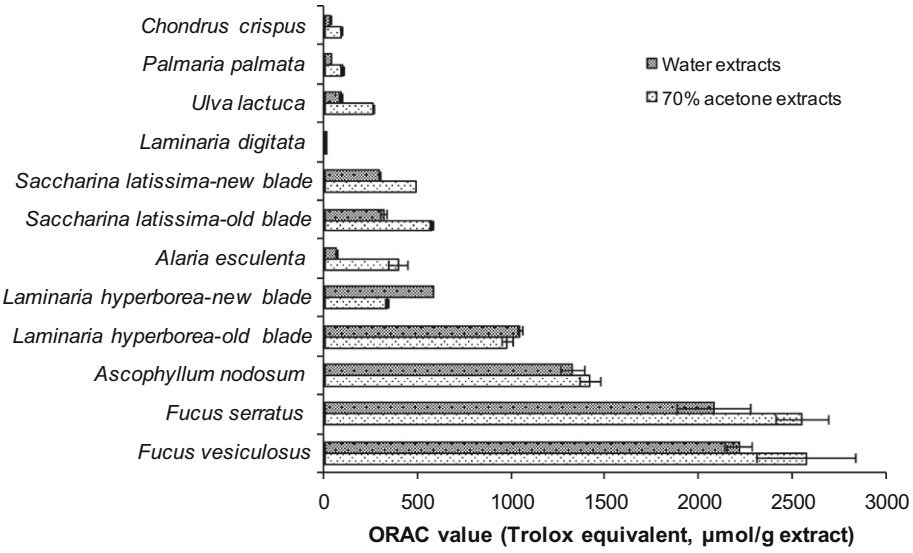


Fig. 3. Oxygen radical absorbance capacity (ORAC) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds. Values are means ± S.D. (n = 3).

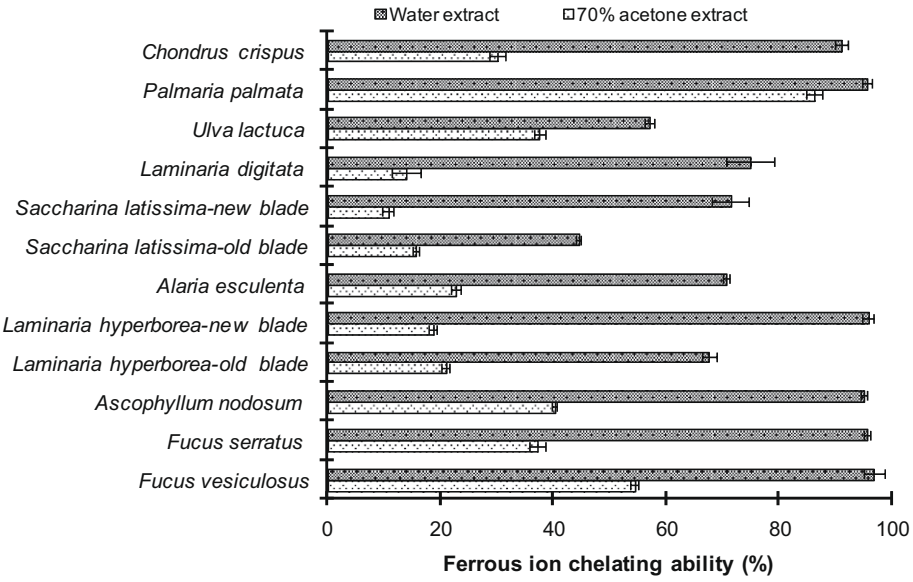


Fig. 4. Ferrous ion-chelating activities of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds. Values are means ± S.D. (n = 3).

3.4. Correlations between TPC and different antioxidant activity assay

PCA was carried out to gain an overview of the similarities and differences among the 10 algal species and to investigate the relationships among TPC and different antioxidant activity assays. The first two principal components explained 74% and 25% of the total variance in the data set, respectively (Figs. 5 and 6). PC1 showed high correlation with TPC, ORAC and DPPH radical scavenging activity. Accordingly, three fucoid seaweeds (*F. vesiculosus, F. serratus* and *A. nodosum*) with the highest TPC, DPPH radical scavenging activities and ORAC values were located furthest to the right along PC1 (Fig. 5). On the other hand, *L. digitata, P. palmata* and *C. crispus* with rather low levels of TPC as well as weak scavenging activities against DPPH and peroxyl radicals were situated on the opposite side of PC1. It is noted that TPC, ORAC and DPPH radical scavenging activity were closely loaded on PC1 (Fig. 6), indicating that TPC and these two antioxidant properties were highly correlated with each other. PC2 mainly explained variation between samples with regard to ferrous ion-chelating ability. Almost all the water extracts appeared in the positive part of the PC2 due to their relatively high ferrous ion-chelating capacity whereas 70% acetone extracts, with the exception of *P. palmata*, were all located in the negative part of the PC2. In addition, ferrous ion-chelating capacity loaded heavily on the second component while TPC has low loading, which illustrates well that no clear correlation exists between TPC and chelating capacity and thus phenolic compounds do not appear to be effective metal chelators (Fig. 6).

High and significant correlation between TPC and DPPH radical scavenging activity of seaweed extracts was further demonstrated by Pearson correlation analysis (WE: r^2 = 0.99; AE: r^2 = 0.99). Similarly, a high correlation was also obtained between TPC and ORAC value (WE: r^2 = 0.96; AE: r^2= 0.99). Previous studies have shown that phenolic compounds are the main contributors to the antioxidant activity of various seaweeds. A positive correlation has been documented between TPC and antioxidant activity of different seaweed extracts by many researchers (Athukorala, Kim, & Jeon, 2006;

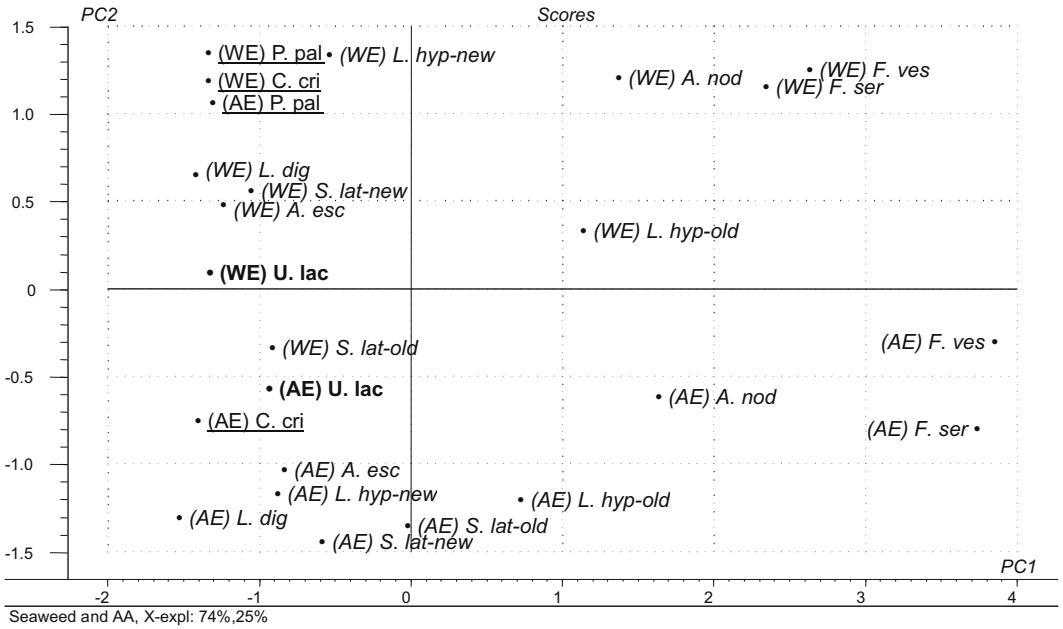


Fig. 5. PCA score plot of total phenolic content (TPC) and antioxidant activities (AA) (DPPH, ORAC and ferrous ion-chelating activity) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds (F. ves, *F. vesiculosus*; F. ser, *F. serratus*; A. nod, *A. nodosum*; L. hyp, *L. hyperborea*; A. esc, *A. esculenta*; S. lat, *S. latissima*; L. dig, *L. digitata*; U. lac, *U. lactuca*; P. pal, *P. palmata*; C. cri, *C. crispus*). Brown seaweeds are indicated by italics; red seaweeds by underline; green seaweeds in bold font.

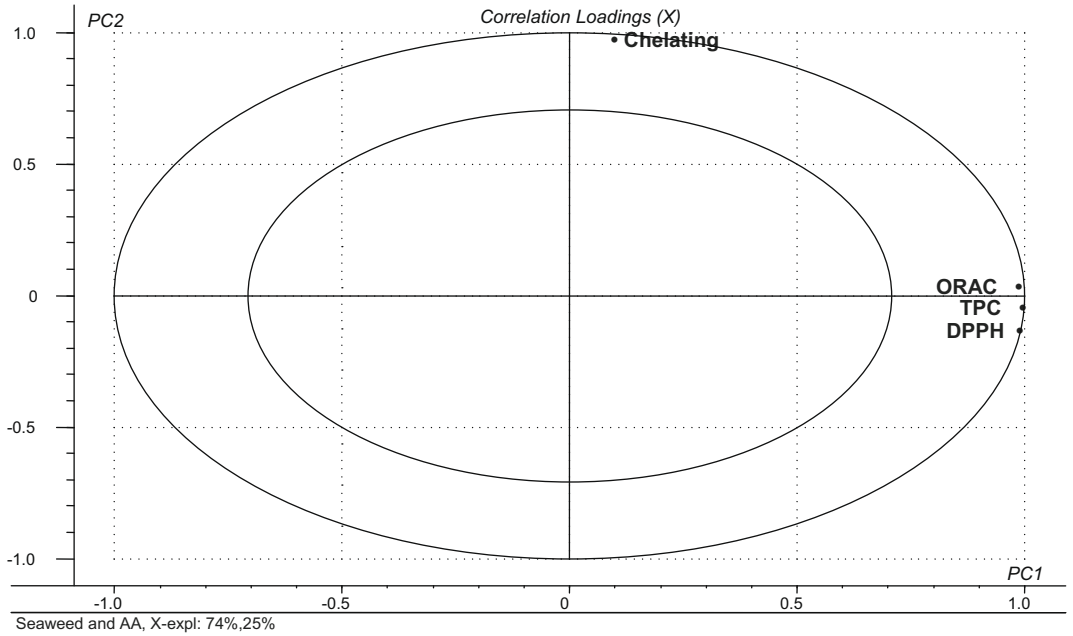


Fig. 6. PCA loading plot of total phenolic content (TPC) and antioxidant activities (AA) (DPPH, ORAC and ferrous ion-chelating activity) of water (WE) and 70% acetone extracts (AE) from selected Icelandic seaweeds.

Jiménez-Escrig et al., 2001; Karawita et al., 2005). A study on the phenolic composition of brown algae *Eisenia bicyclis* by HPLC analyses revealed that the crude polyphenol fraction (ethyl acetate subfraction of methanol extract) was mainly composed of phlorotannin compounds (more than 82%) as well as small amounts of other unknown compounds (Shibata et al., 2004). Preliminary results from a study in our laboratory using similar procedures for the fractionation of *F. vesiculosus* extract showed that the polyphenol-enriched fraction (ethyl acetate fraction) possessed higher DPPH radical scavenging activity than any other fractions as well as the original crude extract (unpublished data). Therefore, phlorotannins, the most abundant group of polyphenols in these seaweeds, appear to be the major contributors to the high scavenging activities. However, this needs to be verified by further characterisation of the extracts. Other active components in different seaweed extracts may have synergistic effects on the scavenging activities, which can give contradictory results. Therefore the assumed role of polyphenols as being mainly responsible for scavenging abilities may be overestimated. Heo, Park, Lee, and Jeon (2005) reported that some enzymatic extracts of *E. cava* and *S. coreanum* exhibited only weak DPPH radical scavenging activities, even though they contained the same level of TPC as other extracts

with higher antiradical activities. Other unknown compounds such as low-molecular-weight polysaccharides, proteins or peptides could also contribute to the scavenging effect.

Interestingly, ferrous ion-chelating ability of water and 70% acetone extracts correlated neither with TPC, nor with DPPH or ORAC (r^2 ranging from 0.17 to 0.46). A similar result was reported by Zhao et al. (2008) who stated that the ferrous ion-chelating ability of malting barley extracts exhibited poor correlations with both TPC and other antioxidant activities (DPPH radical scavenging activity, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation radical scavenging activity and reducing power).

PLSR modelling of the antioxidant properties vs. TPC of seaweed extracts yielded high multivariate correlation coefficient of 0.98 with low RMSEP (1.11). The first two PLSR components explained 99% of the *x*-variables (antioxidant properties) and 98% of the *y*-variables (TPC), respectively. The analysis of the β-coefficients of the variables showed that both DPPH radical scavenging activity and ORAC had high regression coefficient (0.500 and 0.496, respectively) whereas coefficient for ferrous ion-chelating was low (0.033).

Based on these analyses, it can be speculated that the major role of algal polyphenols is as potent radical scavengers and primary, chain-breaking antioxidants. In contrast to what has been reported by Chew et al. (2008), our results showed that algal polyphenols are probably not strong chelators of transition metals. Other components such as polysaccharides, proteins or peptides might be more important for the observed chelating effects of the extracts. However, further study is needed to elucidate the mechanism of antioxidant action of different compounds in the seaweed extracts.

It should also be noted that the reaction conditions of the different antioxidant assays may have a great influence on the effectiveness of various antioxidants. Therefore, to provide further evidence for feasible application of algal polyphenols as natural antioxidants in food for example seafood, the antioxidant potentials of algal polyphenols measured by DPPH, ORAC and metal chelating assays need to be confirmed under appropriate conditions (temperature and pH) of fish muscle system (Decker, Warner, Richards, & Shahidi, 2005).

4. Conclusions

The results of this screening experiment demonstrated that the different Icelandic seaweed species contained different levels of TPC and possessed diverse antioxidant properties. The type of extractant had great impact both on TPC and antioxidant activity of seaweed extracts. 70% acetone was more efficient for polyphenol extraction than water. In general, high TPC correlated with high DPPH and ORAC values, indicating that algal polyphenols were mainly responsible for the free radical scavenging activities of the extracts. However, other co-extracted active compounds such as fucoxanthin and sterols in 70% acetone extracts and sulphated polysaccharides, proteins or peptides in water extracts may also contribute to the overall scavenging effect. Three fucoid species (*F. vesiculosus, F. serratus* and *A. nodosum*) with the highest TPC exhibited the greatest scavenging activities and have therefore be selected for further studies in our laboratory. Interestingly, no clear correlation was found between TPC and chelating ability and thus algal polyphenols did not appear to be effective metal chelators in this experiment. Although the antioxidant activities of the crude seaweed extracts have not been demonstrated herein to be superior to standard compounds (BHT, α-tocopherol and EDTA-Na_2), further fractionation and purification of active components will most likely improve the activity and other potential health benefits may promote their use as natural sources of antioxidants. Future studies will include optimization of extraction, fractionation, purification and characterisation of the active components of these fucoid species. Antioxidant studies in model systems will also be performed to gain a better understanding of their relative antioxidant contribution and mechanisms of action to support the development of seaweed based natural antioxidants for application in functional foods and nutraceuticals.

Acknowledgements

This work was financed by AVS R&D Fund and Icelandic Research Fund and in collaboration with Hollusta úr hafinu ehf. The author Tao Wang would like to thank United Nations University-Fisheries Training Programme for a PhD scholarship granted. Special thanks to Karl Gunnarsson for identification of the seaweed species.

References

Ahn, G. N., Kim, K. N., Cha, S. H., Song, C. B., Lee, J., Heo, M. S., et al. (2007). Antioxidant activities of phlorotannins purified from *Ecklonia cava* on free radical scavenging using ESR and H_2O_2-mediated DNA damage. *European Food Research and Technology, 226*(1–2), 71–79.

Andjelkovic, M., Camp, J. V., Meulenaer, B. D., Depaemelaere, G., Socaciu, C., Verloo, M., et al. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chemistry, 98*(1), 23–31.

Athukorala, Y., Kim, K. N., & Jeon, Y. J. (2006). Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food and Chemical Toxicology, 44*(7), 1065–1074.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Food Science and Technology Lebensmittel-Wissenschaft und Technologie, 28*, 25–30.

Chen, Y., Cai, L., Zhao, C., Xu, H. C., Cao, C. Y., Liu, Y., et al. (2008). Spectroscopic, stability and radical-scavenging properties of a novel pigment from gardenia. *Food Chemistry, 109*(2), 269–277.

Chew, Y. L., Lim, Y. Y., Omar, M., & Khoo, K. S. (2008). Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT – Food Science and Technology, 41*(6), 1067–1072.

Chirinos, R., Rogez, H., Campos, D., Pedreschi, R., & Larondelle, Y. (2007). Optimization of extraction conditions of antioxidant phenolic compounds from mashua (*Tropaeolum tuberosum* Ruíz & Pavón) tubers. *Separation and Purification Technology, 55*(2), 217–225.

Connan, S., Delisle, F., Deslandes, E., & Ar Gall, E. (2006). Intra-thallus phlorotannin content and antioxidant activity in Phaeophyceae of temperate waters. *Botanica Marina, 49*(1), 39–46.

Davalos, A., Gomez-Cordoves, C., & Bartolome, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry, 52*(1), 48–54.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring Antioxidant Effectiveness in Food. *Journal of Agricultural and Food Chemistry, 53*(10), 4303–4310.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry, 38*(3), 674–677.

Elisia, I., Hu, C., Popovich, D. G., & Kitts, D. D. (2007). Antioxidant assessment of an anthocyanin-enriched blackberry extract. *Food Chemistry, 101*(3), 1052–1058.

Galland-Irmouli, A. V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., et al. (1999). Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse). *Journal of Nutritional Biochemistry, 10*(6), 353–359.

Hagerman, A. E. (1988). Extraction of tannin from fresh and preserved leaves. *Journal of Chemical Ecology, 14*(2), 453–461.

He, Y. H., & Shahidi, F. (1997). Antioxidant activity of green tea and its catechins in a fish meat model system. *Journal of Agricultural and Food Chemistry, 45*(11), 4262–4266.

Hemat, R. A. S. (2007). Fat and muscle dysfunction. In R. A. S. Hemat (Ed.), *Andropathy* (pp. 83–85). Dublin, Ireland: Urotext.

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology, 96*(14), 1613–1623.

Ho, S. C., Tsai, T. H., Tsai, P. J., & Lin, C. C. (2008). Protective capacities of certain spices against peroxynitrite-mediated biomolecular damage. *Food and Chemical Toxicology, 46*(3), 920–928.

Huang, D. J., Ou, B. X., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53*(6), 1841–1856.

Huang, D. J., Ou, B. X., Woodill, M. H., Flanagan, J. A., & Prior, R. L. (2002). High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural of Food Chemistry, 50*(16), 4437–4444.

Hultin, H. O. (1994). Oxidation of lipids in seafoods. In F. Shahidi & J. R. Botta (Eds.), *Seafoods: Chemistry, Processing Technology and Quality* (pp. 49–74). Glasgow, UK: Blackie Academic and Professional.

Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R., & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture, 81*(5), 530–534.

Jittrepotch, N., Ushio, H., & Ohshima, T. (2006). Effects of EDTA and a combined use of nitrite and ascorbate on lipid oxidation in cooked Japanese sardine (*Sardinops melanostictus*) during refrigerated storage. *Food Chemistry, 99*(1), 70–82.

Jónsdóttir, R., Bragadóttir, M., & Ólafsdóttir, G. (2007). The role of volatile compounds in odor development during hemoglobin-mediated oxidation of cod muscle membrane lipids. *Journal of Aquatic Food Products Technology, 16*(4), 67–86.

Jormalainen, V., & Honkanen, T. (2004). Variation in natural selection for growth and phlorotannins in the brown alga *Fucus vesiculosus*. *Journal of Evolutionary Biology, 17*(4), 807–820.

Jormalainen, V., Honkanen, T., Vesakoski, O., & Koivikko, R. (2005). Polar extracts of the brown alga *Fucus vesiculosus* (L.) reduce assimilation efficiency but do not deter the herbivorous isopod Idotea baltica (Pallas). *Journal of Experimental Marine Biology and Ecology, 317*(2), 143–157.

Kallithraka, S., Garcia-Viguera, C., Bridle, P., & Bakker, J. (1995). Survey of solvents for the extraction of grape seed phenolics. *Phytochemical Analysis, 6*(5), 265–267.

Karawita, R., Siriwardhana, N., Lee, K. W., Heo, M. S., Yeo, I. K., Lee, Y. D., et al. (2005). Reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition properties of different solvent fractions from *Hizikia fusiformis*. *European Food Research and Technology, 220*(3–4), 363–371.

Kobayashi, M., & Sakamoto, Y. (1999). Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. *Biotechnology Letters, 21*(4), 265–269.

Koivikko, R., Loponen, J., Honkanen, T., & Jormalainen, V. (2005). Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *Journal of Chemical Ecology, 31*(1), 195–212.

Koivikko, R., Loponen, J., Pihlaja, K., & Jormalainen, V. (2007). High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. *Phytochemical Analysis, 18*(4), 326–332.

Miyashita, K., & Hosokawa, M. (2008). Beneficial health effects of seaweed carotenoid, fucoxanthin. In C. Barrow & F. Shahidi (Eds.), *Marine nutraceuticals and functional foods* (pp. 297–319). Boca Raton, FL: CRC Press/Taylor & Francis Group.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*(7), 933–956.

Richards, M. P., & Hultin, H. O. (2000). Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *Journal of Agricultural and Food Chemistry, 48*(8), 3141–3147.

Richards, M. P., & Hultin, H. O. (2002). Contributions of blood and blood components to lipid oxidation in fish muscle. *Journal of Agricultural and Food Chemistry, 50*(3), 550–564.

Rupérez, P., Ahrazem, O., & Leal, J. A. (2002). Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *Journal of Agricultural and Food Chemistry, 50*(4), 840–845.

Saiga, A., Tanabe, S., & Nishimura, T. (2003). Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. *Journal of Agricultural and Food Chemistry, 51*(12), 3661–3667.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture, 76*(2), 270–276.

Santoso, J., Yoshie, Y., & Suzuki, T. (2002). The distribution and profile of nutrients and catechins of some Indonesian seaweeds. *Fisheries Science, 68*(supplement), 1647–1648.

Santoso, J., Yoshie-Stark, Y., & Suzuki, T. (2004). Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. *Fisheries Science, 70*(1), 183–188.

Senevirathne, M., Kim, S. H., Siriwardhana, N., Ha, J. H., Lee, K. W., & Jeon, Y. J. (2006). Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. *Food Science and Technology International, 12*(1), 27–38.

Shibata, T., Kawaguchi, S., Hama, Y., Inagaki, M., Yamaguchi, K., & Nakamura, T. (2004). Local and chemical distribution of phlorotannins in brown algae. *Journal of Applied Phycology, 16*(4), 291–296.

Su, L., Yin, J. J., Charles, D., Zhou, K. Q., Moore, J., & Yu, L. L. (2007). Total phenolic contents, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, rosehip, cinnamon and oregano leaf. *Food Chemistry, 100*(3), 990–997.

Toth, G., & Pavia, H. (2000). Lack of phlorotannin induction in the brown seaweed *Ascophyllum nodosum* in response to increased copper concentrations. *Marine Ecology Progress Series, 192*, 119–126.

Tsai, T. H., Tsai, T. H., Chien, Y. C., Lee, C. W., & Tsai, P. J. (2008). *In vitro* antimicrobial activities against cariogenic streptococci and their antioxidant capacities: A comparative study of green tea versus different herbs. *Food Chemistry, 110*(4), 859–864.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2005). The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chemistry, 93*(4), 713–718.

Undeland, I., Hultin, H. O., & Richards, M. P. (2003). Aqueous extracts from some muscles inhibit hemoglobin-mediated oxidation of cod muscle membrane lipids. *Journal of Agricultural and Food Chemistry, 51*(10), 3111–3119.

Waterman, P. G., & Mole, S. (1994). *Analysis of phenolic plant metabolites (Methods in ecology)*. Oxford, UK: Blackwell Scientific Publications.

Yan, X. J., Chuda, Y., Suzuki, M., & Nagata, T. (1999). Fucoxanthin as the major antioxidant in *Hizikia fusiformis*, a common edible seaweed. *Bioscience, Biotechnology and Biochemistry, 63*(3), 605–607.

Yomauchi, R., Tatsumi, Y., Asano, M., Kato, K., & Ueno, Y. (1988). Effect of metal salts and fructose on the autoxidation of methyl linoleate in emulsions. *Agricultural and Biological Chemistry, 52*(3), 849–850.

Yoshie-Stark, Y., Hsieh, Y. P., & Suzuki, T. (2003). Distribution of flavonoids and related compounds from seaweeds in Japan. *Journal of Tokyo University of Fisheries, 89*, 1–6.

Yoshie, Y., Wang, W., Petillo, D., & Suzuki, T. (2000). Distribution of catechins in Japanese seaweeds. *Fisheries Science, 66*(5), 998–1000.

Zhang, Q., Zhang, J. Z., Shen, J. K., Silva, A., Denis, D. A., & Barrow, C. J. (2006). A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. *Journal of Applied Phycology, 18*(3–5), 445–450.

Zhao, H. F., Fan, W., Dong, J. J., Lu, J., Chen, J., Shan, L. J., et al. (2008). Evaluation of antioxidant activities and total phenolic contents of typical malting barley varieties. *Food Chemistry, 107*(1), 296–304.

Zhao, X., Xue, C. H., & Li, B. F. (2008). Study of antioxidant activities of sulfated polysaccharides from *Laminaria japonica*. Journal of Applied Phycology, DOI: 10.1007/s10811-007-9282-4.

Zhou, K., & Yu, L. (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *Lebennsmittel-Wissenschaft und-Technologie, 37*(7), 717–721.

PAPER III

Enzyme-Enhanced Extraction of Antioxidant Ingredients from Red Algae *Palmaria palmata*

Tao Wang, Rósa Jónsdóttir, Hörður G. Kristinsson, Guðmundur Óli Hreggviðsson, Jón Óskar Jónsson, Guðjón Þorkelsson, Gudrún Ólafsdóttir

III

Submitted for publication

Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*

Tao Wang [1,2,3,*], Rósa Jónsdóttir [2], Hordur G. Kristinsson [2,4], Gudmundur Oli Hreggvidsson [2,5], Jón Óskar Jónsson[2], Gudjon Thorkelsson[1,2] and Gudrún Ólafsdóttir [5,6]

[1]Faculty of Food Science and Nutrition, School of Health Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

[2]Matís ohf., Skulagata 4, 101 Reykjavik, Iceland

[3]Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

[4]Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL.USA

[5]School of Engineering and Natural Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

[6]Syni Laboratory Service, Lyngháls 3, 110 Reykjavik, Iceland

*Corresponding Author

Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

Tel: +86-411-84763557

Fax: +86-411-84763508

Email: wang_tao633@yahoo.com

Abstract

The effects of various enzyme treatments on the extraction of antioxidant ingredients from the red algae *Palmaria palmata* (dulse) were investigated using eleven commercial enzymes including six proteases (Umamizyme, Alcalase, Protamex, Kojizyme, Neutrase and Flavourzyme) and five carbohydrases (Viscozyme, Ultraflo, AMG, Celluclast and Termamyl). Considerable differences were observed both in total phenolic content (TPC) and antioxidant activities of the hydrolysates from *P. palmata* evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, oxygen radical absorbance capacity (ORAC) and ferrous ion-chelating ability assays. All the proteases tested exhibited greater capacities to enhance the extraction of polyphenols and other active components than carbohydrases and cold water extraction (control). The Umamizyme extract had the highest TPC and consequently exhibited the strongest scavenging capacity against DPPH and peroxyl radicals. Further fractionation of the Umamizyme extract revealed that the crude polyphenol fraction possessed the highest peroxyl radical scavenging activity, whereas the crude polysaccharide fraction was more effective for chelating ferrous ions. The data from this study suggest the potential of protease treatment to improve value-added utilization of dulse extracts as antioxidants in functional foods and nutraceuticals.

Keywords: *Palmaria palmata*; Enzymatic hydrolysis; Total phenolic content (TPC); Antioxidant activity; DPPH radical scavenging activity; ORAC; Ferrous ion-chelating ability

1. Introduction

Marine macroalgae have a long history of use as food and folk medicine in Asia, but are much less common in Europe and North America, as a part of the diet. Only a few species are harvested for direct human consumption in limited coastal regions. *P. palmata* (dulse) is one of the most widely distributed edible seaweeds in Iceland and was an important source of food supply when food variety was scarce in earlier times (Kristjánsson, 1980). Nowadays, dulse is mainly consumed as snacks and sold in health stores, but there is growing awareness that it should be utilized more as a source of important nutrients and functional ingredients. It has been reported that dulse has the second highest protein content of all common seaweeds, after *Porphyra tenera* (Nori). The essential amino acids (EAAs) can represent between 26 and 50% of the total amino acids (Galland-Irmouli et al., 1999; Morgan, Wright, & Simpson, 1980). Dulse is also high in iron and many other easily assimilated minerals and trace elements as well as a good source of dietary fibers and vitamins (Morgan et al., 1980).

In recent years, there is increasing interest in the search for naturally occurring compounds with antioxidant activity as alternatives to synthetic products. Aquatic plants are also a rich source of natural antioxidants. Previous studies have shown that *P. palmata* contains several classes of hydrophilic antioxidant components including L-ascorbic acid, glutathione (GSH), polyphenols as well as MAAs (Yuan, Carrington, & Walsh, 2005b; Yuan, Westcott, Hu, & Kitts, 2009). However, the high degree of structural complexity and rigidity of the algal cell wall is a major obstacle to the efficient extraction of the intracellular bioactive constituents (Deniaud, Quemener, Fleurence, & Lahaye, 2003). Conventional water and solvent extraction have several drawbacks such as low selectivity, low extraction efficiency, solvent residue and environmental pollution. As an alternative technology, enzyme-assisted extraction has attracted considerable interest. The application of hydrolytic enzymes has shown a great potential to improve the extraction yield, enhance the release of secondary plant metabolites and preserve the bioactive properties of the extracts. Enzymatic extraction has also been reported to increase the extractability of bioactive compounds from several brown algae (Heo, Park, Lee, & Jeon, 2005; Siriwardhana et al., 2008). These cell wall-degrading enzymes help to weaken or disrupt the cell wall structure, break down complex interior storage materials, thereby facilitating the release of the intracellular bioactive compounds from algal biomass. Furthermore, the hydrolytic breakdown of high-molecular-weight (HMW) polysaccharides and proteins may contribute to enhanced antioxidant activities (Siriwardhana et al., 2008).

In order to promote value-added utilization of this abundant red seaweed and improve the antioxidant activity of the extract, the suitability of different commercial enzymes for effective extraction of water-soluble antioxidant ingredients from *P. palmata* were evaluated herein. The antioxidant properties of different enzymatic extracts were assessed by three *in vitro* assays based upon different reaction mechanisms (DPPH radical scavenging activity, ORAC and ferrous ion-chelating ability).

The antioxidant tests were also carried out on three subfractions from Umamizyme extract, namely crude polyphenol, crude polysaccharide and a LMW aqueous fraction to estimate their relative contribution to the overall antioxidant capacity of the hydrolysate.

2. Materials and methods

2.1. Algal materials

The red algae (Rhodophyta) *Palmaria palmata* (Linnaeus) Kuntze, was collected in Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland on October 12th, 2007. The samples were carefully rinsed with tap water, the small cut pieces were freeze-dried, ground to fine powder and stored in tightly sealed polystyrene containers at −20 °C prior to extraction.

2.2. Chemicals

Fluorescein sodium salt (FL), 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (Ferrozine), Folin–Ciocalteu's phenol reagent were obtained from Fluka (Buchs, Switzerland). 2,2´-azobis (2-methylpropionamidine) dihydrochloride (AAPH) was purchased from Sigma–Aldrich (Steinheim, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was from Sigma–Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

The following eleven commercial enzymes were used for the preparation of hydrolysates, including six types of proteases (Umamizyme, Alacalase 2.4L FG, Protamex, Kojizyme 500 MG, Neutrase 0.8 L and Flavourzyme 500 MG) and five types of carbohydrases (Viscozyme L, Ultraflo L, AMG 300 L, Celluclast 1.5 L FG and Termamyl 120 L). Umamizyme was obtained from Amano Enzyme Inc. (Nagoya, Japan). The other ten enzymes were kindly provided by Novozymes A/S (Bagsvaerd, Denmark). The optimum hydrolysis conditions (Heo et al., 2005; Sato et al., 2002), characteristics and sources of these enzymes are summarized in Table 1.

2.3. Proximate composition analyses of dried P. palmata

Water content was determined gravimetrically by heating the sample in an oven at 103 ± 2 °C for 4 h (ISO 1999). Total fat was determined by extraction with petroleum ether, boiling range 40 °C to 60 °C using an extraction apparatus 2050 Soxtec Avanti Automatic System (AOCS 1998). Protein content was determined by the Kjeldahl method (ISO 2005) and ash according to ISO 5984 (ISO 2002). Percentage of total carbohydrates was determined by subtracting the sum percentage of moisture, crude protein, crude fat and ash.

2.4. Preparation of enzymatic extracts

The enzymatic extracts were prepared according to the method of Heo, Lee, Song and Jeon (2003) with slight modifications. Briefly, 2 g of dried algal sample was dispersed in 50 ml of distilled water

and incubated in a water bath shaker for 10 min. After adjusting pH, 100 mg of enzyme was added. The enzymatic hydrolysis was performed under optimal conditions (Table 1) of the particular enzyme for 24 h (Heo et al., 2005; Sato et al., 2002). The reaction was terminated by boiling the sample at 100 °C for 10 min and thereafter immediate cooling in an ice bath. The hydrolysate was centrifuged at 5,000 *g* for 10 min at 4 °C, filtered with Whatman no. 4 filter paper and the pH of the filtrates was adjusted to pH 7.0. The supernatants were lyophilized, weighed and stored at -20 °C until analyzed. The extraction yield (amount of extractable substances) was expressed as g dried extract/100 g dried algal powder. The extraction was conducted in triplicate. A water extract (2 g algal powder extracted with 50 ml of distilled water for 24 h at room temperature) served as a control.

2.5. Preparation of crude polyphenol, crude polysaccharide and LMW aqueous fractions from Umamizyme hydrolysate

Ten grams of dried Umamizyme extract (prepared by the same procedure described above) were resuspended in 50 ml distilled water, extracted 5 times with 10 ml ethyl acetate (EtOAc). The EtOAc phase was collected. The remaining aqueous layer was precipitated with 99.5 % ethanol (EtOH) (1:3, v/v) at 4 °C for 2 h, followed by centrifugation at 10,000 × g for 20 min. The precipitate was redissolved in distilled water, treated with Sevag reagent (Staub, 1965) several times to remove protein and again precipitated with 3 volumes of EtOH, washed sequentially with absolute ethanol, acetone and ether, then lyophilized to yield the crude polysaccharide extract. The supernatant was concentrated to a small volume with a rotary evaporator and re-extracted with EtOAc. The two EtOAc fractions were combined, evaporated under reduced pressure and then freeze-dried to yield the crude polyphenol extract. Residual solvent in the aqueous phase was removed under vacuum and then redissolved in distilled water, ultrafiltrated at 4 °C by using Centricon Plus-70 centrifugal filter devices (5 kDa nominal molecular weight limit (NMWL), Millipore Corp., Billerica, MA, USA) to remove any residual macromolecules. The filtrates were lyophilized to obtain a LMW aqueous fraction. The preparation procedure was repeated three times.

Three subfractions, crude polyphenol, crude polysaccharide and a LMW aqueous fraction, derived from Umamizyme hydrolysate were analyzed for chemical composition. Soluble protein content was determined according to the Bradford microassay protocol with bovine serum albumin (BSA) as the calibration standard (Bradford, 1976). Total soluble carbohydrates and reducing sugars were analyzed colorimetrically by the modified phenol–sulfuric acid (Masuko, Minami, Iwasaki, Majima, Nishimura, & Lee, 2005) and dinitrosalicylic acid (DNS) (Miller, 1959) methods, respectively. In each assay, glucose was used as the standard.

2.6. Determination of total phenolic content

The TPC of enzymatic extracts and subfractions from Umamizyme hydrolysate were quantified according to the method of Turkmen, Sari and Velioglu (2005) with minor modifications. One

milliliter aliquot of extract solution (concentration range from 1 to 5 mg/ml) was mixed with 5 ml of Folin-Ciocalteu reagent (10% in distilled water). After 5 min, 4 ml of sodium carbonate (7.5% in distilled water) were added. The samples were incubated for 2 h at room temperature in the darkness. The absorbance was measured at 725 nm. A standard curve with serial gallic acid solutions was used for calibration. Results were expressed as gram of gallic acid equivalents (GAE) per 100 g of extract.

2.7. DPPH radical scavenging activity assay

The DPPH radical scavenging activity was estimated according to Sánchez-Moreno, Larrauri and Saura-Calixto (1998) with minor modifications. Briefly, 0.1 ml of the sample solution with different concentrations (at least 5 different concentrations were prepared for each extract/fraction) was mixed with 3.9 ml DPPH• solution (64 μM or 2.5×10^{-2} g/l in methanol, prepared daily). After incubation for 2 h at room temperature, the absorbance was measured at 515 nm. L-ascorbic acid, BHT and α-tocopherol were used as reference standards. The EC_{50} value was calculated as the concentration of sample or standard antioxidant (mg/ml) required to scavenge 50% of the DPPH• in the reaction mixture. Its reciprocal, the antiradical power (ARP, $ARP = 1/EC_{50}$) was also calculated.

2.8. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to Davalos, Gomez-Cordoves and Bartolome (2004) with slight modifications (Wang, Jónsdóttir, & Ólafsdóttir, 2009). Mx300 real-time PCR System (Stratagene Inc., La Jolla, CA) was used for the fluorescence measurements controlled by MxPro computer program. All reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). Ten microliters of each sample solution (concentration range from 0.025 to 0. 5 mg/ml) was mixed with 60 μl of 70 nM fluorescein and pre-incubated at 37 °C for 15 min. The reaction was initiated by addition of 30 μl of 12 mM AAPH solution. The fluorescence (FAM) was recorded every 0.1 min for the first 20 cycles and every minute thereafter until the fluorescence of the last reading declined to <5% of the initial value. Excitation and emission filter wavelengths were set at 484 nm and 520 nm, respectively. The ORAC value was calculated and expressed as micromoles of Trolox equivalents per gram extract (μmol of TE/g extract) using the calibration curve of Trolox.

2.9. Ferrous ion-chelating ability assay

The ferrous ion-chelating ability was determined according to the method of Decker and Welch (1990) with minor modifications. One hundred microliters of sample solution (5 mg/ml) were mixed with 135 μl of distilled water and 5 μl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 10 μl of 5 mM ferrozine. After incubation for 10 min at room temperature, the absorbance was measured at 562 nm. Distilled water (100 μl) instead of sample solution was used as a control. Distilled water (10 μl) instead of ferrozine solution was used as a blank. L-ascorbic acid, citric acid and EDTA-Na_2 were used as reference standards. The ferrous ion-chelating ability was calculated as follows:

$$\text{Ferrous ion-chelating ability}(\%) = [(A_0 - (A_1 - A_2)]/A_0 \times 100.$$

where A_0 was the absorbance of the control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.10. Statistical analysis

All tests were conducted in triplicate. Analysis of variance (ANOVA) followed by Duncan's test was carried out to test for differences between different enzyme treatments and between different fractions from Umamizyme hydrolysate in the statistical program NCSS 2000 (NCSS, Kaysville, Utah, USA). Significance of differences was defined at the 5% level ($p < 0.05$).

3. Results and discussion

3.1. Proximate composition of P. palmata

Proximate composition analyses showed that the dried *P. palmata* sample contained approximately 4.1 ± 0.4 % moisture, 61.5 ± 2.4 % carbohydrate, 20.5 ± 0.4 % crude protein, 0.4 ± 0.4 % crude fat and 13.5 ± 0.5 % ash. The crude protein content was within the range for *P. palmata* (8-35%) as reported earlier by Morgan et al. (1980) and Galland-Irmouli et al. (1999). Significant seasonal fluctuations of protein content in *P. palmata* were previously reported by Galland-Irmouli et al. (1999) and Fleurence (1999). Higher protein levels were recorded for samples collected during winter and spring whereas lower amounts were observed during summer and early autumn. The percentage concentrations of carbohydrate, crude fat and ash in the dried *P. palmata* sample were in agreement with previous studies (Morgan et al., 1980).

3.2. Extraction yield of enzymatic hydrolysates from P. palmata

All the commercial enzymes tested in this study were effective in improving the extraction yield, but to different extent compared with water extraction (Fig. 1). Umamizyme gave the highest yield (76.3 ± 1.4 %), approximately 2 times higher than water extraction (37.8 ± 1.9 %), followed by Ultraflo (68.3 ± 1.5 %) and Celluclast (66.6 ± 2.2 %).

3.3. Effects of different enzyme treatments on the extraction of total phenolics from P. palmata

Considerable variation was observed in TPC among different enzymatic hydrolysates, ranging from 0.3 to 1.6 g GAE/100 g extract (Fig. 2). All the proteases tested showed greater capacities to enhance the recovery of phenolic compounds than water extraction. On the contrary, the TPC of the hydrolysates extracted by carbohydrases (except Viscozyme) were generally lower. Particularly, Umamizyme exhibited the highest extraction efficiency of phenolic compounds compared to other proteases and carbohydrases tested, which was approximately 3.0-fold higher than that of water extract.

The relatively lower TPC of both water and carbohydrase extracts could be partly due to the formation of protein–polyphenol complexes during the extraction. When the algal cell wall is disrupted, the intracellular constituents including proteins are released from the cells, which are prone to complex with polyphenols, leading to aggregation and ultimate precipitation (Siriwardhana et al., 2008). Many studies have been performed to investigate the mechanisms of polyphenol-protein interactions. Potentially, polyphenols may interact with proteins via hydrogen bonding, π-bonding, hydrophobic interactions, ionic and covalent linkage. Hydrogen bonding and hydrophobic interactions were reported as the dominant modes of interaction between tannins and proteins (Carvalho, De Freitas, & Mateus, 2008; Hagerman, 1992; Shahidi & Naczk, 2004). Although much less information is available in the literature regarding the complexation of algal polyphenols with proteins, strong interactions between phlorotannins (the largest group of polyphenols present in brown algae) and proteins were reported by Stern, Hagerman, Steinberg and Mason (1996). Therefore, the use of proteases substantially convert proteins into small peptides and free amino acids and reduce the complex formation between protein and algal polyphenols, which could explain the improved extraction efficiency of phenolic compounds by proteases observed in the present study. Another explanation for the decreased TPC in carbohydrase extracts could be due to the release of oligosaccharides and simple sugars during the degradation of cell wall polysaccharides.

3.4. Effects of different enzyme treatments on the antioxidant properties of P. palmata

3.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity of enzymatic hydrolysates increased in a concentration-dependent manner and also increased with the increment of the incubation time (data not shown).

As shown in Fig. 3, all of the hydrolysates obtained by protease treatments, except for Alcalase extract, exhibited higher scavenging effect than that of water extract. In particular, Umamizyme treatment significantly increased the radical scavenging capacity (ARP = 1.8, EC_{50} = 0.6 mg/ml), which was around 3.4 times higher than that of water extract (ARP = 0.5, EC_{50} = 1.9 mg/ml). On the contrary, all the hydrolysates extracted by carbohydrases showed relatively weaker scavenging activity than the control sample, with ARP values all below 0.3.

The higher radical scavenging capacity of protease extracts could be due to their ability to improve the extraction efficiency of phenolic compounds which have been reported to be potent DPPH radical scavengers. Moreover, the liberation of LMW peptides and amino acids by proteases might also contribute to the enhanced scavenging activities of the extracts. In contrast to other fungal proteinases, Umamizyme not only has high protease activity, but also has high peptidase activity. The Umamizyme hydrolysate of tuna stomach proteins was reported to contain high proportion of small soluble peptides below 5 KDa (Guerard, Guimas, & Binet, 2002). Several studies have shown that LMW hydrolysates generally possess higher DPPH radical scavenging capacity than HMW hydrolysates (Chang, Wu, & Chiang, 2007; Wu, Chen, & Shiau, 2003).

All the reference compounds, L-ascorbic acid (ARP = 400.9, $EC_{50} = 2.5\times10^{-3}$ mg/ml), BHT (ARP = 305.2, $EC_{50} = 3.3\times10^{-3}$ mg/ml) and α-tocopherol (ARP = 168.6, $EC_{50} = 5.9\times10^{-3}$ mg/ml), exhibited much higher DPPH radical scavenging activity when compared with all the enzymatic hydrolysates tested. This is in agreement with previous studies by Yuan, Bone and Carrington (2005a), who found that the DPPH radical scavenging activity of 1-butanol soluble fraction from methanol extract of *P. palmata* was hundreds of times weaker than those of L-ascorbic acid and BHA.

3.4.2. ORAC

Further evaluation of the chain-breaking antioxidant activities of different enzymatic hydrolysates and water extract was conducted by ORAC-FL assay. The peroxyl radical scavenging activity of water extract was 35.8 μmol TE/g extract (Fig. 4), which was comparable to the reported value for methanol extract (36.42 to 38.78 μmol TE/g extract) of *P. palmata* from the west and east coasts of Grand Manan Island, New Brunswick, Canada (Yuan et al., 2009). Enzyme treatments by all the proteases and Viscozyme (carbohydrase) resulted in enhanced peroxyl radical scavenging capacity. Umamizyme was the most efficient enzyme, showing the greatest increase in ORAC value (4.2 fold) as compared to its water extract counterpart, followed by Alcalase (3.9 fold) and Neutrase (3.0 fold). The higher ORAC value of protease extracts could be attributed to the improved extraction efficiency of phenolic compounds as well as the liberation of LMW peptides and amino acids.

3.4.3. Ferrous ion-chelating activity

All the hydrolysates demonstrated the ability to chelate ferrous ions at concentrations of 5 mg/ml (Fig. 5). However, their activities were all lower than that of water extract (95.6 % at 5 mg/ml). Enzyme treatments by Celluclast, AMG and Viscozyme of carbohydrases and Umamizyme of proteases resulted in significant decreases in chelating capacity.

The high heavy metal binding capacity of algal dietary fibers such as alginate, fucoidan from brown algae and carrageenan, agar from red algae is well documented. The enzymatic degradation of these soluble and insoluble dietary fibers into oligosaccharides and simple sugars might reduce the chelating capacity to different extent (Fig. 5), depending on the composition and structure of degradation products. In addition, some peptides as well as proteins have also been reported to possess the ability to chelate metal ions. The lower chelating ability of Umamizyme extract in this study indicated that HMW peptides and proteins might be more effective ferrous ion chelators than LMW peptides and amino acids. It is also worth noting that no clear correlation was found between TPC and chelating capacity of *P. palmata* hydrolysates (data not show), supporting our previous finding that algal polyphenols were not effective as metal chelators (Wang et al., 2009).

The chelating capacity of three reference antioxidants including L-ascorbic acid, citric acid and EDTA-Na_2 was also tested for comparison. All the enzymatic extracts exhibited higher chelating activity than those of L-ascorbic acid (8.2% at 20 mg/ml) and citric acid (21.5% at 5 mg/ml). However,

their activity was much lower than that of the potent metal-ion chelator, EDTA-Na_2 (99.8% at 0.05 mg/ml).

3.5. Approximate chemical composition and antioxidant activities of crude polyphenol, crude polysaccharide and LMW aqueous fractions from Umamizyme extract

The TPC of the crude polyphenol fraction from the Umamizyme extract was 5.7 g GAE/100 g extract, while the TPC of the crude polysaccharide and LMW aqueous fraction (<5 KDa) was 1.0 and 1.4 g GAE/100 g extract, respectively (Table 2). The levels of total soluble carbohydrates, reducing sugars and soluble protein were analyzed in the crude polysaccharide and LMW aqueous fractions, as shown in Table 2.

The DPPH radical scavenging activity of three fractions from Umamizyme extract decreased in the following order: crude polyphenol > crude polysaccharide > LMW aqueous fraction (Table 3). The order of ORAC values was: crude polyphenol > LMW aqueous fraction > crude polysaccharide fraction. However, crude polysaccharide fraction exhibited the highest ferrous ion-chelating ability, followed by crude polyphenols and the smallest effect was found in LMW aqueous fraction. The relatively strong scavenging effects of the crude polyphenol fraction on DPPH and peroxyl radicals confirmed the key role of algal polyphenols as free radical scavengers and primary, chain-breaking antioxidants, as suggested in our previous study (Wang et al., 2009). HMW polysaccharides contributed greatly to the ferrous ion-chelating and DPPH quenching activities of the hydrolysate. However, their peroxyl radical scavenging capacity was weaker than the other two fractions. LMW peptides, amino acids as well as some of the mycosporine-like amino acids (MAAs) might be the major contributors for the moderate radical scavenging capacity of LMW aqueous fraction. It should also be noted that the antioxidant activity of this fraction might not accurately reflect the real antioxidant potentials of LMW peptides and MAAs because of the presence of large amount of other water soluble components (such as simpler sugars) with insignificant antioxidant activity in this fraction (Table 2).

4. Conclusions

The results of the present study showed that enzyme-assisted extraction was effective in enhancing the recovery of polyphenols and other hydrophilic antioxidant compounds from *P. palmata*. Proteases were more effective than carbohydrases and Umamizyme extract exhibited the greatest scavenging activity against DPPH and peroxyl radicals. The use of different *in vitro* antioxidant tests verified the complexity of the antioxidant effects of the seaweed hydrolysates Crude polyphenols contributed greatly to the peroxyl radical scavenging properties of Umamizyme extract whereas crude polysaccharides appeared to be more potent metal chelators in this experiment. Treatment of *P. palmate* by Umamizyme improves versatile application of extracts as natural antioxidants and

functional food ingredients. For application in food as natural antioxidants, their effect has to be further studied in relevant food matrices.

Acknowledgement

This work was financed by AVS R&D Fund of Ministry of Fisheries in Iceland and Icelandic Research Fund and in collaboration with Hollusta úr hafinu ehf. The author Tao Wang would like to thank United Nations University-Fisheries Training Programme for a PhD scholarship granted.

References

AOCS, Official method BA 3-38. (1998). Application note Tecator nr AN 301. In D. Firestone, *Official methods and recommended practices of the American Oil Chemists' Society* (5th ed.) (pp. 4). Champaign, IL: American Oil Chemists' Society.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248-254.

Carvalho, E., De Freitas, V., & Mateus, N. (2008). Overview of protein-tannin interactions. In B. Pignataro, *Tomorrow's chemistry today: Concepts in nanoscience, organic materials and environmental chemistry*, (pp. 412-415). Weinheim, Germany: Wiley-VCH.

Chang, C. Y., Wu, K. C., & Chiang, S. H. (2007). Antioxidant properties and protein compositions of porcine haemoglobin hydrolysates. *Food Chemistry*, 100 (4), 1537-1543.

Davalos, A., Gomez-Cordoves, C., & Bartolome, B. (2004). Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. *Journal of Agricultural and Food Chemistry*, 52 (1), 48–54.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38 (3), 674–677.

Deniaud, E., Quemener, B., Fleurence, J., & Lahaye, M. (2003). Structural studies of the mix-linked β-(1 → 3)/β-(1 → 4)-D-xylans from the cell wall of *Palmaria palmata* (Rhodophyta). *International Journal of Biological Macromolecules*, 33 (1-3), 9-18.

Fleurence, J. (1999). Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science and Technology*, 10 (1), 25-28.

Galland-Irmouli, A. V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., et al. (1999). Nutritional value of proteins from edible seaweed *Palmaria palmata* (Dulse). *Journal of Nutritional Biochemistry*, 10 (6), 353–359.

Guerard, F., Guimas, L., & Binet, A. (2002). Production of tuna waste hydrolysates by a commercial neutral protease preparation. *Journal of Molecular Catalysis B: Enzymatic*, 19–20, 489–498.

Hagerman, A. E. (1992). Tannin-protein interactions. In C. T. Ho, C. Y. Lee, M. T. Huang, *Phenolic compounds in food and their effects on health I. Analysis, occurrence, and chemistry*, ACS Symposium Series (Vol. 506) (pp. 236–247). Washington, DC: American Chemical Society.

Heo, S. J., Lee, K. W., Song, C. B., & Jeon, Y. J. (2003). Antioxidant activity of enzymatic extracts from brown seaweeds. *Algae*, 18 (1)**,** 71-81.

Heo, S. J., Park, E. J., Lee, K. W., & Jeon, Y. J. (2005). Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresource Technology*, 96 (14), 1613-1623.

ISO (1999). *Animal feeding stuffs - Determination of moisture and other volatile matter content*, (ISO 6496) (pp. 7). Geneva, Switzerland: International Organization for Standardization.

ISO (2002). *Animal feeding stuffs - Determination of crude ash*, (ISO 5984) (pp. 6). Geneva, Switzerland: International Organization for Standardization.

ISO (2005). *Animal feeding stuffs - Determination of nitrogen content and calculation of crude protein content - Part 2: Block digestion/steam distillation method*, (ISO 5983-2) (pp. 14). Geneva, Switzerland: International Organization for Standardization.

Kristjánsson, L. (1980). Söl. In L. Kristjánsson, *Íslenskir sjávarhættir 1*, (pp. 42-83). Reykjavík, Iceland: Menningarsjóður.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol–sulfuric acid method in microplate format. *Analytical Biochemistry*, 339 (1), 69-72.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, 31 (3), 426–428.

Morgan, K. C., Wright, J. L. C., & Simpson, F. J. (1980). Review of chemical constituents of the red alga *Palmaria palmata* (Dulse). *Economic Botany*, 34 (1), 27–50.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 76 (2), 270–276.

Sato, M., Oba, T., Yamaguchi, T., Nakano, T., Kahara, T., Funayama, K., et al. (2002). Antihypertensive effects of hydrolysates of wakame (*Undaria pinnatifida*) and their angiotensin I-converting enzyme inhibitory activity. *Annals of Nutrition and Metabolism*, 46 (6), 259-267.

Shahidi, F. & Naczk, M. (2004). Nutritional and pharmacological effects of food phenolics. In F. Shahidi, M. Naczk, *Phenolics in food and nutraceuticals*, (pp. 331-402). Boca Raton, FL: CRC Press.

Siriwardhana, N., Kim, K. N., Lee, K. W., Kim, S. H., Ha, J. H., Song, C. B. et al. (2008). Optimisation of hydrophilic antioxidant extraction from *Hizikia fusiformis* by integrating treatments of enzymes, heat and pH control. *International Journal of Food Science and Technology*, 43 (4), 587-596.

Staub, A. M. (1965). Removal of protein-Sevag method. In R. L. Whistler, J. N. BeMiller, M. L. Wolfrom, *Methods in carbohydrate chemistry*, *general polysaccharide.* (Vol. 5) (pp. 5-6). New York: Academic Press.

Stern, J. L., Hagerman, A. E., Steinberg, P. D., & Mason, P. K. (1996). Phlorotannin–protein interactions. *Journal of Chemical Ecology*, 22 (10), 1877–1899.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2005). The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chemistry*, 93 (4), 713-718.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic Seaweeds. *Food Chemistry*, 116 (1), 240-248.

Wu, H. C., Chen, H. M., & Shiau, C. Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food Research International*, 36 (9-10), 949–957.

Yuan, Y. V., Bone, D. E., & Carrington, M. F. (2005a). Antioxidant activity of dulse (*Palmaria palmata*) extract evaluated in vitro. *Food Chemistry*, 91 (3), 485-494.

Yuan, Y. V., Carrington, M. F., & Walsh N. A. (2005b). Extracts from dulse (*Palmaria palmata*) are effective antioxidants and inhibitors of cell proliferation in vitro. *Food and Chemical Toxicology*, 43 (7), 1073–1081.

Yuan, Y. V., Westcott, N. D., Hu, C., & Kitts, D. D. (2009). Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. *Food Chemistry*, 112 (2), 321-328.

Figure Captions

Fig. 1. Extraction yield of different enzymatic extracts from *P. palmata* compared to water extract (WE). The extraction yield was expressed as g dried extract/100 g dried algal powder. Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

Fig. 2. Total phenolic content (TPC) of different enzymatic extracts from *P. palmata* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

Fig. 3. DPPH radical scavenging activity (expressed as antiradical power (ARP); $ARP=1/EC_{50}$) of different enzymatic extracts from *P. palmata* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

Fig. 4. Oxygen radical absorbance capacity (ORAC) of different enzymatic extracts from *P. palmata* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

Fig. 5. Ferrous ion-chelating ability of different enzymatic extracts from *P. palmata* compared to water extract (WE). Values are means ± S.D. (n = 3). Protease extracts are indicated by dark gray bars; Carbohydrase extracts by light gray bars; Water extract by white bars.

Table 1 Summary of the optimum hydrolysis conditions, characteristics and sources of specific enzyme

Enzyme[a]	Optimum conditions[b]		Characteristics	Sources
	pH	Temperature (°C)		
Umamizyme	7.0	50	Endo- and exo-peptidase complex	*Aspergillus oryzae*
Alacalase 2.4L FG	8.0	50	Endo-peptidase	*Bacillus licheniformis*
Protamex	6.0	40	Protease complex	*Bacillus* sp.
Kojizyme 500 MG	6.0	40	Amino- and carboxy-peptidase	*Aspergillus oryzae*
Neutrase 0.8 L	6.0	50	Metallo-endoprotease	*Bacillus amyloliquefaciens*
Flavourzyme 500 MG	7.0	50	Endo-protease and exo-peptidase	*Aspergillus oryzae*
Viscozyme L	4.5	50	A multi-enzyme complex (containing arabanase, cellulase, β-glucanase, hemicellulase and xylanase)	*Aspergillus aculeatus*
Ultraflo L	7.0	60	Heat-stable multi-active β-glucanase	*Humicola insolens*
AMG 300 L	4.5	60	Exo-1,4-α-D-glucosidase	*Aspergillus niger*
Celluclast 1.5 L FG	4.5	50	Cellulase	*Trichoderma reesei* ATCC 26921
Termamyl 120 L	6.0	60	Heat-stable α-amylase	*Bacillus licheniformis*

[a] Umamizyme was obtained from Amano Enzyme Inc. (Nagoya, Japan). The other enzymes were from Novozymes A/S (Bagsvaerd, Denmark).

[b] Heo et al. (2005); Sato et al. (2002).

Table 2 Content of total phenolics, total soluble carbohydrates, reducing sugars and soluble proteins in crude polyphenol, polysaccharide and LMW aqueous fractions from Umamizyme extract of *P. palmata*

Sample	TPC[a] (g GAE/100 g)	Total soluble carbohydrates[b] (g Glu/kg)	Reducing sugars (g Glu/kg)	Soluble protein (g /kg)
Crude polyphenol fraction	5.7 ± 0.2	nd[c]	nd	nd
Crude polysaccharide fraction	1.0 ± 0.1	579.9 ± 21.3	182.1 ± 4.2	7.5 ± 0.2
LMW aqueous fraction (<5 KDa)	1.4 ± 0.0	405.0 ± 17.5	325.2 ± 10.9	0.6 ± 0.0

Each value is expressed as means ± S.D. (n = 3).

[a] TPC, total phenolic content, g gallic acid equivalents (GAE) /100g extract.

[b] Glu, glucose.

[c] nd, not detected due to lack of quantity.

Table 3 DPPH radical scavenging activity, ORAC and ferrous ion-chelating ability of crude polyphenol, polysaccharide and LMW aqueous fractions from Umamizyme extract of *P. palmata*

Sample	DPPH scavenging[a] (ARP)	ORAC[b] (μmol TE/g extract)	Fe^{2+} chelating ability (%)
Crude polyphenol fraction	5.4 ± 0.3	629.5 ± 15.2	57.2 ± 2.9[c]
Crude polysaccharide fraction	4.8 ± 0.3	102.9 ± 2.8	94.3 ± 1.8[c]
LMW aqueous fraction (<5 KDa)	2.4 ± 0.1	130.7 ± 3.4	20.5 ± 1.6[d]

Each value is expressed as means ± S.D. (n = 3).

[a] ARP, antiradical power (ARP=1/EC_{50}), EC_{50}: concentration of extract (mg/ml) required to scavenge 50% of the DPPH• in the reaction mixture.

[b] ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents.

[c] was tested at concentration of 2.5 mg/ml.

[d] was tested at concentration of 5.0 mg/ml.

Figure 1.

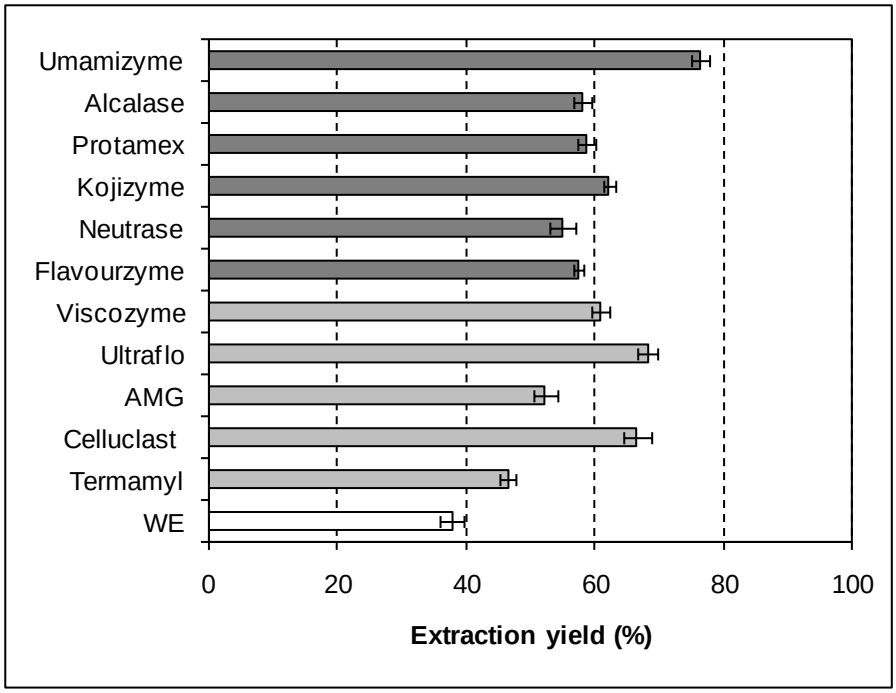


Figure 2.

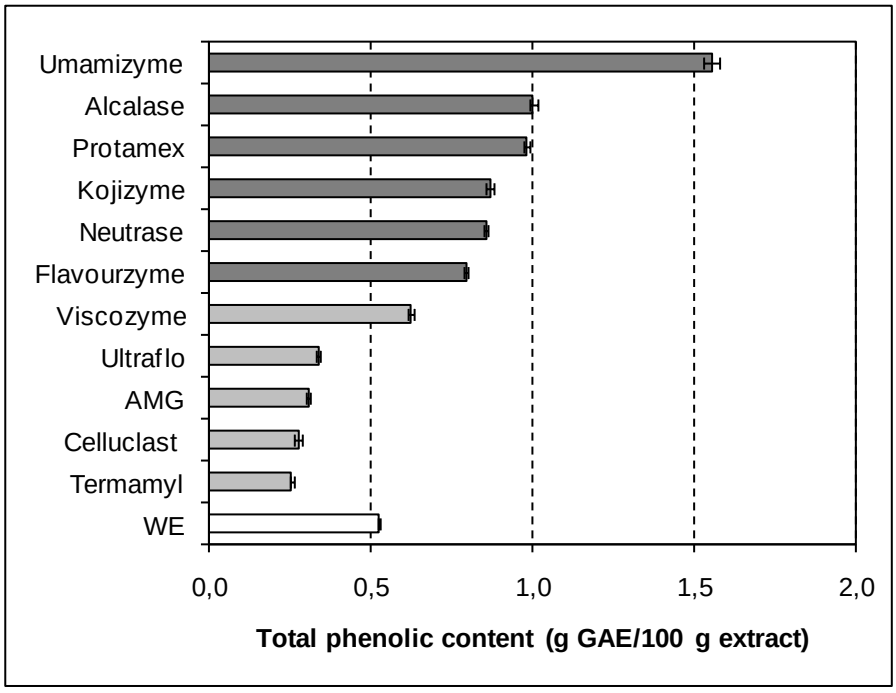


Figure 3.

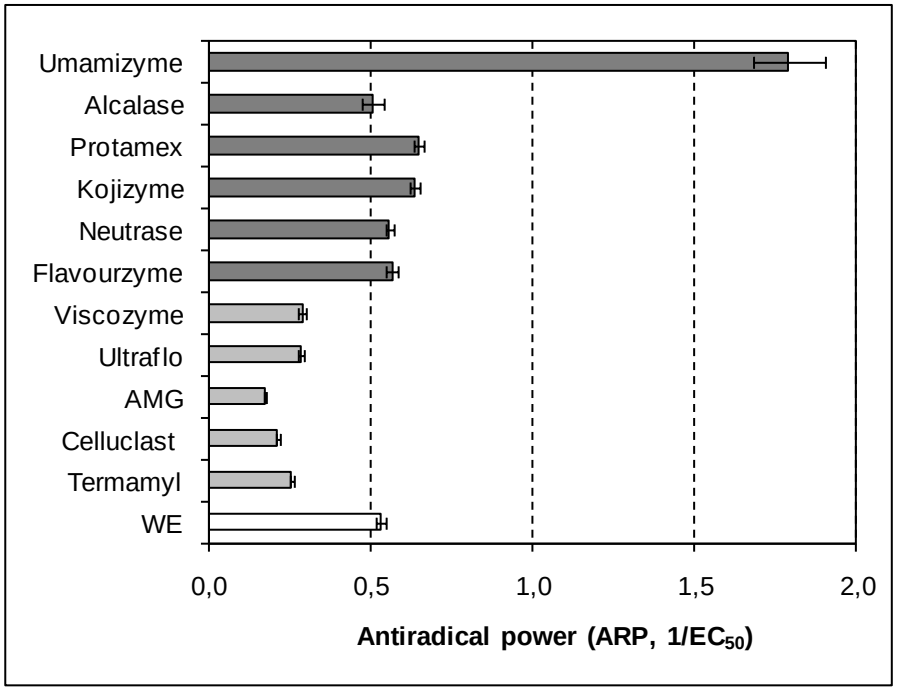


Figure 4.

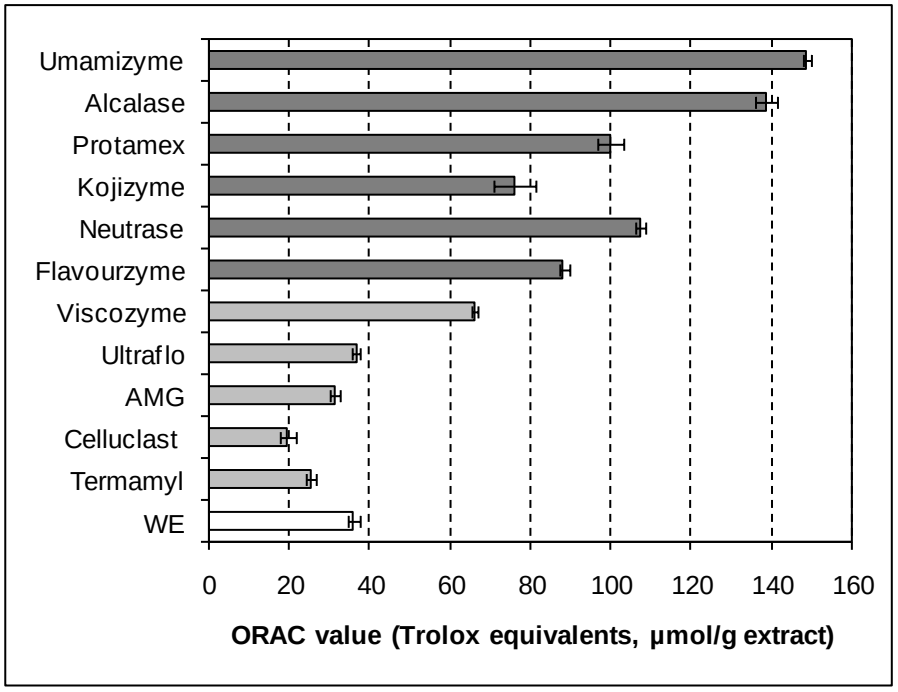
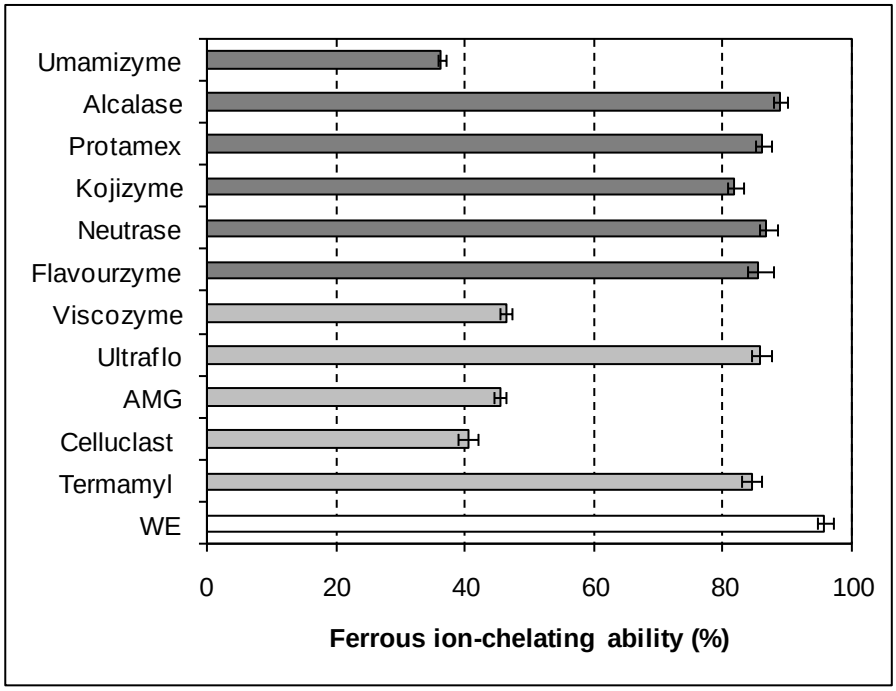


Figure 5.


Umamizyme
Alcalase
Protamex
Kojizyme
Neutrase
Flavourzyme
Viscozyme
Ultraflo
AMG
Celluclast
Termamyl
WE
0
20
40
60
80
100
Ferrous ion-chelating ability (%)

PAPER IV

Antioxidant Properties of Solvent Extracts and Fractions from the Brown Algae *Fucus vesiculosus*

Tao Wang, Rósa Jónsdóttir, María Jesús González, Isabel Medina, Hörður G. Kristinsson, Sivakumar Raghavan, Guðrún Ólafsdóttir

IV

Manuscript

Antioxidant properties of solvent extracts and fractions from the brown algae *Fucus vesiculosus*

Tao Wang [1,2,3,*], Rósa Jónsdóttir [2], María Jesús González [4], Isabel Medina [4], Hordur G. Kristinsson [2,5], Sivakumar Raghavan [5] and Gudrún Ólafsdóttir [6]

[1]Faculty of Food Science and Nutrition, School of Health Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

[2]Matís ohf., Skulagata 4, 101 Reykjavik, Iceland

[3]Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

[4]Instituto de Investigaciones Marinas del CSIC. Eduardo Cabello 6, E-36208 Vigo, Spain

[5]Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL, USA

[6]School of Engineering and Natural Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

*Corresponding Author

Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

Tel: +86-411-84763557

Fax: +86-411-84763508

Email: wang_tao633@yahoo.com

Abstract

A process for the effective extraction and fractionation of phlorotannins from *F. vesiculosus* with high antioxidant potentials was investigated. Antioxidant activity of *F. vesiculosus* extract/fractions was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, reducing power and ferrous ion-chelating assays. Among the crude extract and different polarity fractions, phlorotannin-enriched ethyl acetate (EtOAc) fraction possessed the highest DPPH scavenging activity and reducing power. This fraction was further fractionated by Sephadex LH–20 column chromatography or ultra-filtration. The antioxidant properties were evaluated by both above chemical antioxidant tests and a mononuclear cell-based bioassay. Sephadex subfractions LH–2 and LH–3 with high total phlorotannin content (TPC) exhibited strong DPPH quenching activity, comparable to ascorbic acid and butylated hydroxytoluene (BHT) and significantly higher than α-tocopherol. Polyphenols in *F. vesiculosus* were found to consist mainly of high molecular weight (HMW) phlorotannin polymers. There were no clear relationships between the degree of polymerization, molecular size and antioxidant activities. All the subfractions separated by Sephadex LH-20 column and ultra-filtration showed high ability to scavenge reactive oxygen species (ROS) generated by mononuclear cells. HPLC-DAD-MASS analysis showed that the phlorotannins with molecular weight (MW) < 2 KDa in EtOAc fraction was mainly composed of 3 (3PL) to 5 (5PL) units of phloroglucinol.

Keywords: *Fucus vesiculosus*; Phlorotannin compounds; Fractionation; Antioxidant; DPPH; Reducing power; Ferrous ion-chelating; Reactive oxygen species; Mononuclear cells

1. Introduction

Currently there is an increased awareness and safety concern towards synthetic antioxidants and a worldwide trend to apply natural antioxidants to replace synthetic compounds as additives in foods or as nutraceuticals and functional food ingredients (Balasundram, Sundram, & Samman, 2006; Kim, Rajapakse, & Shahidi, 2007). Natural antioxidants derived from various plants and marine algae not only show great potential for improving oxidative stability of food products, but also have a broad array of additional health promoting benefits. An appropriate intake of dietary antioxidants has been suggested to play an important role in enhancing the body's defense system and preventing ROS-related diseases (Halliwell, 1996; Halliwell & Gutteridge, 2007).

Marine macroalgae have been an important part of the human diet in Asia since ancient times and are traditionally well renowned for their versatile health benefits. Recent studies have revealed that marine algae are not only a good source of dietary fibre, proteins, vitamins and several minerals, but also contain a large array of secondary metabolites with valuable biological activities, which cannot be found in terrestrial plants (Plaza, Cifuentes, & Ibanez, 2008). A number of potent antioxidant compounds have been isolated and identified from different types of seaweeds, including phlorotannins, sulphated polysaccharides, carotenoid pigments such as fucoxanthin and astaxanthin, sterols, catechins and mycosporine-like amino acids (MAAs) (Miyashita & Hosokawa, 2008; Rupérez, Ahrazem, & Leal, 2002; Yuan, Westcott, Hu, & Kitts, 2009). Phlorotannins, the dominant polyphenolic secondary metabolites found only in brown algae (Phaeophyta), have recently attracted considerable research interest because of their superior antioxidant activities. Phlorotannins are formed by the polymerisation of phloroglucinol (1,3,5-trihydroxybenzene) monomer units and biosynthesised via the acetate–malonate pathway, also known as polyketide pathway (Ragan & Glombitza 1986). Based on the type of linkage between the phloroglucinol sub-units as well as the number of additional hydroxyl groups on the aromatic skeletons, phlorotannins can be classified into six major subclasses: phlorethols, fucols, fuhalols, fucophlorethols, isofuhalols and eckols (Ragan et al., 1986; Targett & Arnold, 1998). Several species of brown algae such as *Ecklonia cava*, *Ecklonia kurome*, *F. vesiculosus*, *Hizikia fusiformis* and *Sargassum ringgoldianum* have been found to possess remarkably high antioxidant activity *in vitro*, which is well correlated with their content of TPC (Nakai, Kageyama, Nakahara, & Miki, 2006; Senevirathne, Kim, Siriwardhana, Ha, Lee, & Jeon, 2006). The superior antioxidant properties have also been reported for individual phlorotannin compounds, including eckol, dieckol, phlorofucofuroeckol A and 8,8′-bieckol (Shibata, Ishimaru, Kawaguchi, Yoshikawa, & Hama, 2008). The scavenging activities of these phlorotannin compounds on DPPH and superoxide anion radicals were found to be around 2 to 10 times higher than those of catechin, α-tocopherol and ascorbic acid.

Recently, a preliminary experiment was carried out in our laboratory to investigate the antioxidant potentials of ten species of common Icelandic seaweeds (Wang, Jónsdóttir, & Ólafsdóttir, 2009).

Among the screened species, *F. vesiculosus* showed the highest TPC, the strongest scavenging activities against DPPH and peroxyl radicals as well as a moderate ferrous ion-chelating ability. However, no comprehensive study has been performed on the extraction and characterisation of antioxidant properties of phlorotannins from *F. vesiculosus* harvested in Iceland. Therefore, the objective of the present work was to develop a process for effective extraction and fractionation of phlorotannins from *F. vesiculosus*. The antioxidant activity of different extract/fractions was characterised using a multiple-method approach which included well documented *in vitro* antioxidant assays and a mononuclear cell-based bioassay. The information gained will help to understand how molecular size and degree of polymerisation may influence the antioxidant activity and to promote the potential application of phlorotannin-rich extract and fractions as natural antioxidants in food products and in health-promoting functional food formulations.

2. Materials and methods

2.1. Algal materials

The brown algae (Phaeophyta) *Fucus vesiculosus* (Linnaeus) was collected in Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland on October 15th, 2008. The seaweeds were washed with clean seawater to remove epiphytes and sand attached to the surface and transported to the laboratory. The samples were carefully rinsed with tap water. Small pieces were cut and then freeze-dried, pulverised into fine powder and stored in tightly sealed polystyrene containers at −20 °C prior to extraction.

2.2. Chemicals

Folin–Ciocalteu's phenol reagent, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt (Ferrozine), ferric chloride ($FeCl_3$) were purchased from Fluka (Buchs, Switzerland). Iron (II) chloride was obtained from Sigma–Aldrich (Steinheim, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), potassium ferricyanide ($K_3Fe(CN)_6$), phorbol myristate acetate (PMA), horse radish peroxidase (HRP) and 4-aminopthalhydrazide (isoluminol) were from Sigma–Aldrich (St. Louis, MO, USA). All the solvents used were of HPLC grade. All other chemicals and reagents were of analytical grade.

2.3. Preparation of seaweed extracts and fractions

The solvent extracts were prepared according to the method described by Wang et al. (2009). Briefly, 2 g of dried algal powder was dispersed in 50 mL of extraction solvent, including methanol/water (80:20, v/v), ethyl acetate/water (80:20, v/v), acetone/water (70:30, v/v), ethanol/water (80:20, v/v), ethanol/water (50:50, v/v) and incubated in a platform shaker (Innova™ 2300, New Brunswick Scientific, Edison, NJ) for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at

2500 *g* for 10 min at 4 °C and filtered with Whatman no. 4 filter paper. The solvent was removed by rotary evaporation. The residue was lyophilized, weighed and stored at -20 °C until further analysis. The extraction yield (amount of total extractable substances, TES) was expressed as g dried extract/100 g dried algal powder. Each extraction was conducted in triplicate. Cold and hot water extracts were also prepared (2 g algal powder extracted with 50 mL of distilled water for 24 h at 20 °C and 70 °C, respectively) and tested in parallel.

The crude 80% EtOH extract was subjected to solvent fractionation. The dried algal powder (40 g) was extracted with 200 mL 80% EtOH by the same procedure described above. The extract was concentrated *in vacuo* to a small volume and the residue was suspended in a mixture of methanol (MeOH) and water (40:30, v/v) and partitioned three times with *n*-hexane, EtOAc and *n*-butanol (BuOH) successively (Figure 1). After removal of the solvent and freeze-dried, four fractions were obtained, including the *n*-hexane-, EtOAc-, *n*-butanol-soluble fractions and aqueous residue.

The EtOAc-soluble fraction, which showed the highest TPC, DPPH scavenging activity and reducing power, was further subjected to gel filtration on a Sephadex LH–20 column and eluted stepwise with solvent systems of decreasing polarity, yielding six subfractions: LH–1 (50% aqueous MeOH, v/v), LH–2 (75% aqueous MeOH, v/v), LH–3 (pure MeOH), LH-4 (MeOH/acetone 5:1, v/v), LH–5 (MeOH/acetone 3:1, v/v) and LH–6 (MeOH/acetone 1:1, v/v). The solvent in each subfraction was evaporated under reduced pressure and then lyophilized (Figure 1).

2.4. Preparation of different molecular weight subfractions from EtOAc-soluble fraction

In order to characterize the MW distribution, EtOAc-soluble fraction was also sub-fractionated into five fractions: U1 (> 100 kDa), U2 (30–100 kDa), U3 (10–30 kDa), U4 (5–10 kDa) and U5 (< 5 kDa) by using a series of Centricon filters (Centricon Plus-70 centrifugal filter units, nominal molecular weight limit (NMWL) of 100, 30, 10 and 5 kDa, Millipore Corp., Billerica, MA, USA) (Figure 1). Two grams of dried EtOAc-soluble fraction from 80% EtOH crude extract were redissolved in Milli-Q water, first filtered at 15 °C through the Centricon-100 and the fraction with MW >100 kDa was saved. The flow-through was then centrifuged successively over Centricon-30, 10 and 5 filters. All the retentates and <5 kDa filtrate were collected separately and freeze-dried.

2.5. Determination of total phlorotannin content

The TPC of the extract/fractions was quantified according to the method of Turkmen, Sari, and Velioglu (2005) and Koivikko, Loponen, Honkanen, and Jormalainen (2005) with some modifications. One milliliter aliquot of sample solution (concentration range from 0.1 to 8 mg/mL) was mixed with 5 mL of Folin-Ciocalteu reagent (10% in distilled water). After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) were added. The samples were incubated for 2 h at room temperature in the darkness. The absorbance was measured at 725 nm with a UV–vis spectrophotometer (Ultrospec 3000 pro, Amersham Pharmacia Biotech, Ltd., Cambridge, UK). A standard curve with serial

phloroglucinol solutions (20 – 100 μg/mL) was used for calibration. Results were expressed as gram of phloroglucinol equivalents (PGE) per 100 g of extract.

2.6. DPPH radical scavenging activity

The DPPH radical scavenging activity was estimated according to Sánchez-Moreno, Larrauri, and Saura-Calixto (1998) with slight modifications. Briefly, 0.1 mL of the sample solution (at least 5 different concentrations were prepared for each extract/fraction) was mixed with 3.9 mL DPPH• solution (64 μM or 2.5×10^{-2} g/L in methanol, prepared daily). After incubation for 2 h at room temperature, the absorbance was measured at 515 nm with the Ultrospec 3000 pro UV–vis spectrophotometer. L-ascorbic acid, BHT and α-tocopherol were used as reference standards. The calibration curve made with DPPH• between 4.15×10^{-3} and 4.15×10^{-2} g/L was used to calculate the remaining concentration of DPPH• in the reaction medium. The EC_{50} value was calculated as the concentration of sample or standard antioxidant (μg/mL) required to scavenge 50% of the DPPH• in the reaction mixture.

2.7. Reducing power

The reducing power was measured as described by Benjakul, Visessanguan, Phongkanpai, and Tanaka (2005). Fifty microliters of seaweed extract solution (0.1 mg/mL) were mixed with 250 μL of phosphate buffer (0.2 M, pH 6.6) and 250 μL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. After 30 min incubation at 50 °C, 250 μL of 10% trichloroacetic acid was added and the mixture was centrifuged at 4500 *g* for 10 min. The supernatant (200 μL) was mixed with 40 μL of ferric chloride solution (0.1%) and the absorbance was measured at 700 nm. The relative activity of the sample was calculated in relation to the activity of ascorbic acid standards (0-200 μg/mL) and the results were expressed as mg of ascorbic acid equivalents (ASE) per gram of extract.

2.8. Ferrous ion-chelating ability

The ferrous ion-chelating ability was determined according to the method of Decker and Welch (1990) with minor modifications. One hundred microliters of sample solution (5 mg/ml) were mixed with 135 μL of distilled water and 5 μL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 10 μL of 5 mM ferrozine. After incubation for 10 min at room temperature, the absorbance was measured at 562 nm with a Tecan Sunrise microplate reader (Tecan Austria Gesellschaft, Salzburg, Austria). Distilled water (100 μL) instead of sample solution was used as a control. Distilled water (10 μL) instead of ferrozine solution was used as a blank. L-ascorbic acid, citric acid and EDTA-Na_2 were used as reference standards. The ferrous ion-chelating ability was calculated as follows:

$$\text{Ferrous ion-chelating ability}(\%) = [(A_0 - (A_1 - A_2)]/A_0 \times 100.$$

where A_0 was the absorbance of the control, A_1 was the absorbance of the sample or standard and A_2 was the absorbance of the blank.

2.9. Isolation of mononuclear cells from whole blood

Mononuclear cells were isolated from the blood of human volunteers. Whole blood was collected in BD Vacutainer® CPT cell preparation tubes containing sodium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and was immediately mixed by inverting the tubes 8-10 times. The blood samples were then centrifuged at 1500 *g* for 15 min at room temperature (18–25 ^{o}C) to separate the mononuclear cells as a whitish layer beneath the blood plasma. The cells were washed twice with cold phosphate buffered saline solution containing calcium and magnesium salts, and suspended in Krebs-Ringer bicarbonate buffer supplemented with glucose, pH 7.3 (KRG buffer). The number of cells were counted using hematocytometer and the concentration was adjusted to 5 x 10^6 cells/ mL. The percentage of various cells obtained using BD Vacutainer method was reported as 79% lymphocytes and 12% monocytes (Data on file, Report no. R-88-99-QC-195, BD Vacutainer Systems, NJ, USA). All human procedures were approved by the University of Florida Institutional Review Board.

2.10. Chemiluminescence method

Chemiluminescence (CL) technique (Gunnarsson et al., 2006) was used for detecting ROS produced by PMA-stimulated human mononuclear cells. In this method, the reaction mixture contained 200 μL of 51.2 mM isoluminol, 100 μL of 5 units HRP, 2.0 mL of Krebs-Ringer bicarbonate (KRG) buffer and 200 μL of mononuclear cells (5 x 10^5 cells/ mL). The reaction was initiated using 200 μL PMA (10^{-5} M). Subfractions obtained from Sephadex LH–20 chromatography and ultra-filtration were dissolved and diluted in water and added to the reaction mixture to a final concentration of 1.5 μg/ mL reaction mixture and 2.4 μg/ mL reaction mixture respectively. The ability of the seaweed samples to quench ROS was measured as CL using a LS-45 luminescence spectrometer (Perkin Elmer Instruments, Waltham, MA, USA) at 25 ^{o}C. The area under the CL curve was calculated and compared with a control containing only KRG buffer. The ROS scavenging ability of various subfractions were measured by their ability to reduce the area under the CL curve.

2.11. HPLC-DAD-MASS analysis of the EtOAc fraction

HPLC separation was performed on a Hypercarb column (Thermo), 5 μm 4.6 x150 mm. HPLC-DAD-MASS was equipped with a quaternary pump (Spectra System P4000) and a diode array detector (UV 6000LP) coupled to an Ion Trap Mass spectrometer (LCQ DECA XP Plus, Thermo Fisher Scientific) with an electrospray source in negative mode. Chromatographic conditions: The mobile phase was a binary solvent gradient system consisting of (A) HCOOH 0.05% and (B) CH3CN and a flow rate of 0.25 mL/min. The elution profile was as follows: Initial conditions were 5% of B. Isocratic elution during five min. After that, solvent B was increased to 20% in 15 min, then linear for 15 min, subsequently increased to 70% of B in 5 min and then linear for 10 min. Mass spectrometer conditions were: Sheath gas flow rate was 45 units, voltage was 5.16 Kv and capillary temperature was 350 ºC. The EtOAc-soluble fraction was dissolved in MeOH (1 mg/mL).

2.12. Statistical analysis

Each extraction and fractionation experiment was replicated three times and analyses were performed at least two times. Analysis of variance (ANOVA) was applied to the data using the Number Cruncher Statistical Software, NCSS 2000 (NCSS, Kaysville, Utah, USA). Significant differences were determined by one way ANOVA and Tukey-Kramer Multiple-Comparison Test was used to determine the statistical difference between sample groups. Significance of differences was defined at the 5% level ($p<0.05$).

3. Results and discussion

3.1. Effect of solvent type on the extraction of phlorotannins from F. vesiculosus

Based on our preliminary experiment (data not shown) and other studies, polar solvents are more efficient at extracting phlorotannins than water and apolar solvents (Koivikko et al., 2005). Therefore, the focus of the present study was to compare the extraction efficiency of various polar solvent systems.

The type of solvent had an impact on the amount of TES (Table 1). In general, the yield decreased with decreasing polarity of the solvent in the order of water, 50% EtOH, 80% MeOH, 80% EtOH, 70% acetone and 80% EtOAc. The highest TES was recorded for the hot water extract (37.8 ± 0.5) whereas the lowest for 80% EtOAc extract (12.0 ± 0.3).

The influence of the solvent type upon the extraction efficiency and selectivity of phlorotannins was examined. In general, all the polar solvents were more efficient than water and the highest recovery was obtained by using 70% aqueous acetone (Table 1). This is in agreement with the findings of Koivikko et al. (2005) who reported that 70% aqueous acetone was the most efficient solvent for the extraction of phlorotannin compounds from *F. vesiculosus* among eight extractants with different polarities. Hot water extraction resulted in a significantly lower level of TPC than did cold water extraction, which might be due to the thermal decomposition of some phlorotannin compounds at elevated temperatures as well as a significant increase in the extraction of undesired concomitant components. There was no clear relationship between the amount of TES and the TPC of the extracts.

The TPC of all solvent extracts of *F. vesiculosus* in the present study were higher than those reported in the literature, including 70% acetone extract of *F. vesiculosus* from the Finnish Archipelago Sea (30 g PGE /100 g extract on dry weight basis) (Jormalainen, Honkanen, Vesakoski, & Koivikko, 2005) and methanol extract (23.21 g PGE /100 g extract) from Atlantic coast of Canada (Zhang, Zhang, Shen, Silva, Denis, & Barrow, 2006). It was also noted that the TPC values of both cold water and 70% acetone extract were much higher than those reported in our earlier study, in which the sample was collected in early spring (Wang et al., 2009). Significant seasonal fluctuations of TPC in brown fucoid algae are well documented and the variations appear to be related to several environmental factors such as light intensity, ultraviolet radiation and nutrient availability (Pavia & Toth, 2000; Swanson &

Druehl, 2002). Higher TPC level generally occurs in summer and early autumn whereas lower amount is observed during winter, supporting the important photo-protective roles of phlorotannins.

DPPH radical scavenging activity of various extracts was in the following order: 70% acetone > 80% MeOH > 80% EtOH > 80% EtOAc > 50% EtOH > cold water (20°C) > hot water (70°C) (Table 1). It was observed that the antioxidant activity of different extracts in DPPH assay was linearly correlated with TPC level (data not shown).

Since 80% ethanol also showed considerable ability to extract phlorotannins from *F. vesiculosus*, the use of ethanol would obviously be preferred over acetone and methanol for the extraction of food-grade natural antioxidants. 80% EtOH extract was therefore chosen for further fractionation studies.

3.2. Total phlorotannin content and antioxidant activities of 80% ethanol extract and its solvent fractions

The 80% EtOH extract was sequentially separated into four fractions, including the *n*-hexane-, EtOAc-, *n*-BuOH-soluble fractions and the final aqueous residue, by liquid–liquid partition.

There were significant differences in TPC among 80% EtOH extract and its subsequent fractions. The highest level of TPC was found in the EtOAc fraction with the value of 88.3 ± 2.2 g PGE/ 100 g extract. The crude extract, *n*-BuOH fraction and aqueous residue had the TPC values of 35.4 ± 0.2, 62.8 ± 1.3, 12.0 ± 0.7 g PGE/ 100 g extract, respectively. The lowest TPC was observed for *n*-hexane fraction (2.2 ± 0.2 g PGE/ 100 g extract). Ethyl acetate has been widely used to selectively extract polyphenolic compounds of intermediate polarity from various plants (Mariod, Ibrahim, Ismail, & Ismail, 2009; Parejo et al., 2002). The present study showed that it is also effective in concentrating/enriching phlorotannins from the crude extract of *F. vesiculosus*.

As shown in Table 2, four fractions with different polarity scavenged DPPH radicals in a dose-dependent manner over a concentration range of 2–20 μg/mL. The EtOAc-soluble fraction exhibited the highest scavenging activity, followed by *n*-butanol fraction, while the aqueous residue and hexane fraction were much less effective. The activity of EtOAc and *n*-BuOH fractions was comparable to the commercial antioxidants, α-tocopherol, BHT and ascorbic acid. The reducing power of the extract/fractions decreased in the same order of EtOAc fraction (757.7 ± 38.2 mg ASE/g) > *n*-BuOH fraction (487.7 ± 12.6 mg ASE/g) > 80% EtOH extract (324.8 ± 8.9 mg ASE/g) > aqueous residue (109.3 ± 0.6 mg ASE/g). However, all the extract/fractions showed relatively similar chelating ability of 34.7 ± 1.9%, 34.2 ± 1.3%, 30.9 ± 1.6% and 35.1 ± 2.3% for the 80% EtOH extract, EtOAc and *n*-BuOH fractions and aqueous residue, respectively.

In addition, positive correlations between TPC, DPPH radical scavenging activity and reducing power were observed for the 80% EtOH extract and its solvent fractions based on Pearson correlation analysis (data not shown), supporting our previous finding that phlorotannins are the major antioxidant components present in *F. vesiculosus* (Wang et al., 2009). However, there was no relationship between

TPC and ferrous ion-chelating capacity and phlorotannins did not appear to be very effective metal chelators.

3.3. Total phlorotannin content and antioxidant activities of Sephadex LH–20 chromatographic subfractions

The EtOAc-soluble fraction, which possessed the highest TPC as well as the strongest DPPH scavenging activity and reducing power, was further separated by Sephadex LH–20 column chromatography to give six subfractions (LH–1 to LH–6).

Sephadex LH–20 gel has been widely used for the fractionation and purification of various groups of phenolic compounds that occur in plants and marine algae (Amarowicz, Troszynska, & Shahidi, 2005; Kantz & Singleton, 1990; Li, Qian, Ryu, Lee, Kim, & Kim, 2009). The separation is mainly based on hydrogen bonding between phenolic OH- groups and the ether oxygen atoms of the crossing-linking chain of the gel (Kantz et al., 1990; Shahidi & Naczk, 2004). The gel thus functions as a hydrogen bond acceptor and the strength of adsorption depends upon the number of phenolic hydrogens per molecule. Polymeric polyphenols containing many phenolic hydroxyl groups are retained more strongly on the column than monomers and oligomers. Using appropriate eluent system allows separation of non-polymeric and polymeric phenols based on hydrogen-bonding properties (Kantz et al., 1990; Oberholster, 2008). In this study, phlorotannin oligomers were desorbed first from the gel by aqueous methanol. Gradual desorption of polymeric phlorotannins was achieved by stepwise increase in the acetone concentration of acetone–methanol elution mixture. The carbonyl oxygen of acetone has been reported to serve as a strong hydrogen bond acceptor and enables displacement of complexed polymeric phenols from Sephadex LH–20 column (Kantz et al., 1990; Jerez, Tourino, Sineiro, Torres, & Nunez, 2007).

The relative yields of different Sephadex subfractions are shown in Table 3. Approximately 75.4 % of algal material could be recovered after Sephadex LH–20 chromatographic separation. The majority of the phlorotannin components in the EtOAc-soluble fraction were eluted from the column in the last three subfractions and the percentage recovery was 12.7%, 46.2% and 18.9%, respectively. LH–5 was found to be the most abundant fraction in mass while LH–1 was the least abundant. It can therefore be assumed that phlorotannins in *F. vesiculosus* comprise mainly HMW polymers.

The subfractions LH–2 and LH–3 with higher TPC were found to possess better DPPH scavenging activity (EC_{50} value was 2.79 and 2.82 μg/mL, respectively) than the other subfractions and the EtOAc fraction itself (Table 3). The activity was comparable to those of the positive controls, ascorbic acid (EC_{50} = 2.49 μg/mL) and BHT (EC_{50} = 3.28 μg/mL), and was significantly higher than that of α-tocopherol (EC_{50} = 5.93 μg/mL) (Table 2). The TPC and DPPH quenching activity of subfractions LH–4 to LH–6 were similar to that of the original EtOAc fraction, whereas subfraction LH–1 had significantly lower TPC and weaker scavenging activity. Similar trends were observed with reducing power and no direct relationship was found between TPC and metal chelating capacity. In general,

there was no clear trend in antioxidant activity among oligomeric and polymeric subfractions, but the differences in the reducing and DPPH scavenging activities appeared to be explained only by their relative TPC levels. Phlorotannin oligomers and polymers did not seem to differ markedly in antioxidant activity.

Unlike plant-derived polyphenols, the relationship between molecular structure and antioxidant activity of brown algal phlorotannins is still poorly understood. Several lines of evidence indicate that oligomeric and polymeric phlorotannins are more potent antioxidants than the monomer phloroglucinol (Cerantola, Breton, Ar Gall, & Deslandes, 2006; Kim, Lee, Shin, & Lee, 2004; Nakai et al., 2006). Oligomerization of phloroglucinol appears to be crucial for the radical scavenging activity. However, research exploring the correlation between the antioxidant capacity and specific structural features of phlorotannins has yielded contradictory findings. Cerantola et al. (2006) isolated two types of structurally distinct phlorotannins from *Fucus spiralis* extracts and compared their DPPH radical scavenging activity. The first polymer had relatively simple structure and belonged to the fucol subclass, whereas the second one was more complex and linked through aryl-aryl and aryl-ether bonds, thus belonged to the fucophlorethol subclass. Both phlorotannin compounds showed equally high scavenging activity in comparison to ascorbic acid and phloroglucinol monomer, indicating that there was no clear association between the type of structural linkages and radical scavenging activity of phlorotannins. On the other hand, several other studies showed that the presence of O-bridge linkages (ether linkages) and the number and arrangement of phenolic hydroxyl groups in phlorotannin skeleton have profound influence on the antioxidant property. The extraordinary antioxidant potential of specific phlorotannin compounds extracted from Ecklonia species such as *E. cava* and *E. stolonifera* has been ascribed to their unique dibenzo-1,4-dioxin unit in the molecular skeleton (Li et al., 2009; Shin, Hwang, Kang, & Lee, 2006). In another study, several oligomeric phlorotannins such as eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were found to possess varying degrees of scavenging capacity against DPPH and superoxide anion radicals, which was assumed to be related to the number of hydroxyl groups attached to the eckol skeleton (Shibata et al., 2008).

3.4. Total phlorotannin content and antioxidant activities of different molecular weight subfractions

Currently, little information is available concerning the MW distribution of phlorotannins in *F. vesiculosus* as well as the relationship between MW and antioxidant activity. In this study, EtOAc-soluble fraction was sequentially fractionated through ultra-filtration membranes to yield five subfractions: > 100 kDa, 100–30 kDa, 30–10 kDa, 10–5 kDa and < 5 kDa. The antioxidant activity of different subfractions was evaluated by the methods described above.

Subfraction with MW > 100 kDa was found to be the most abundant and represented about 73.9% of the total amount recovered, indicating that highly polymerized phlorotannins are the largest pool of phenolic compounds in *F. vesiculosus*. The yields of subfractions 5–10 kDa and 10–30 kDa were significantly lower than the other subfractions (Table 4). This is consistent with a recent study

showing that the EtOH extract of *F. vesiculosus* was composed of mainly HMW phlorotannin polymers, around 83% of which having a MW of more than 50 kDa (Zaragoza et al., 2008).

As summarized in Table 4, subfractions containing high TPC such as 10–30 kDa, 30–100 kDa and 5–10 kDa, generally exhibited strong DPPH radical scavenging activity and reducing power. There also seemed to be no clear relationship between the molecular size of phlorotannin compounds and *in vitro* antioxidant activity.

3.5. Chemiluminescence assay for the detection of reactive oxygen species

The ability of different subfractions obtained from sequential ultra-filtration and Sephadex LH–20 gel chromatography, to scavenge ROS produced by stimulated human mononuclear cells was studied using an isoluminol-enhanced CL method. The antioxidant activity of the algal samples were measured by their ability to quench ROS (or chromogenic radicals) as reflected by a decrease in chemiluminescence signal. At a concentration of 2.4 μg/mL, all the ultra-filtered subfractions showed a significantly high ($p<0.05$) ability to scavenge ROS compared to the control (Figures 2). The average ROS quenching ability appeared to increase with a decrease in MW, but the difference was not statistically significant ($p>0.05$). Although no study has been performed to assess the ROS scavenging ability of algal phlorotannins in mononuclear cell-based bioassay system, the ability of low MW compounds to quench PMA-induced ROS was also reported by Raghavan, Kristinsson, and Leeuwenburgh (2008) while studying tilapia protein hydrolysates. They reported a higher potential for 25% hydrolyzed proteins (fractions with lower MW) compared to 15% and 7.5% hydrolyzed proteins (high MW proteins) to inhibit PMA-induced CL. The chemiluminescence quenching ability of different MW subfractions was however not reflected in their DPPH radical scavenging activity, reducing power and iron chelating ability (Table 4).

All the gel filtration subfractions also exhibited quenching of PMA induced isoluminol CL (Figure 3). Among the various subfractions, LH–2, LH–5 and LH–6 exhibited significant ($p<0.05$) CL quenching at 1.5 μg/ mL and LH–1 at 3.0 μg/ mL concentration. In accordance with the chemical antioxidant tests, oligomeric and polymeric subfractions did not seem to differ markedly with respect to ROS scavenging ability in mononuclear cells, although these subfractions exhibited different reactivity pattern in different antioxidant assays.

3.6. HPLC-DAD-MASS analysis of the EtOAc faction

In order to gain preliminary information on the major composition of phluoroglucinol units in *F. vesiculosus* extract, HPLC-DAD-MASS analysis was carried out on the phlorotannin-enriched EtOAc fraction. The phlorotannin components identified by HPLC-DAD-MASS were mainly oligomers or polymers with MW < 2 kDa. The results of full mass chromatogram showed that these LMW oligomers or polymers in the EtOAc fraction was composed by units of phloroglucinol (1,3,5-trihydroxybenzene) from 3 (3PL) to 6 (6PL) units. The phlorotannins with 4 (4PL) to 5 (5PL) units

were the most abundant, accounting for 83% of the total phlorotannins identified (data not shown). The bond nature between the monomer units can be C-C or C-O (ether bridges). However, the presence of phloroglucinol monomer was not detected.

4. Conclusions

This study provides initial evidence on the antioxidant activity and possible health benefits of *F. vesiculosus* extract/fractions. The phlorotannin-enriched EtOAc fraction showed the highest reducing and DPPH scavenging capacities among the crude extract and different polarity fractions. High correlations were observed between TPC, DPPH scavenging and reducing capacity, suggesting that phlorotannins are the major antioxidant principles in *F. vesiculosus*. Subfractions rich in oligomeric and polymeric phlorotannins exhibited potent DPPH scavenging activity, comparable to or even higher than several commercial antioxidants. There were no clear correlations between degree of polymerisation, molecular size and chemical antioxidant activities. Moreover, all the subfractions exhibited similarly high scavenging activity towards ROS produced by stimulated human mononuclear cells. Further isolation and structure elucidation of phlorotannin compounds present in different subfractions are needed for a deeper understanding of the mechanisms behind the high antioxidant effects observed herein.

Acknowledgements

This work was financed by AVS R&D Fund of Ministry of Fisheries in Iceland and Icelandic Research Fund and in collaboration with Íslensk hollusta ehf. The author Tao Wang would like to thank United Nations University-Fisheries Training Programme for a PhD scholarship granted.

References

Amarowicz, R., Troszynska, A., and Shahidi, F. (2005). Antioxidant activity of armond seed extract and its fractions. *Journal of Food Lipids*, 12(4), 344–358.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), 191-203.

Benjakul, S., Visessanguan, W., Phongkanpai, V. and Tanaka, M. (2005). Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. *Food Chemistry.* 90(1-2), 231–239.

Cerantola, S., Breton, F., Ar Gall, E., & Deslandes, E. (2006). Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. *Botanica Marina*, 49(4), 347–351.

Decker, E. A. & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674–677.

Gunnarsson, G., Undeland, I., Sannaveerappa, T., Sandberg, A. S., Lindgard, A., Mattsson-Hulten, L., & Soussi, B. (2006). Inhibitory effect of known antioxidants and of press juice from herring (*Clupea harengus*) light muscle on the generation of free radicals in human monocytes. *Journal of Agricultural and Food Chemistry*, 54(21), 8212-8221.

Halliwell, B. (1996). Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radical Research*, 25(1), 57-74.

Halliwell, B., & Gutteridge, J. M. C. (2007). *Free Radicals in Biology and Medicine* (4th ed.). Oxford, UK: Clarendon Press.

Jerez, M., Tourino, S., Sineiro, J., Torres, J. L., & Nunez, M. J. (2007). Procyanidins from pine bark: Relationships between structure, composition and antiradical activity. *Food Chemistry*, 104(2), 518–527.

Jormalainen, V., Honkanen, T., Vesakoski, O., & Koivikko, R. (2005). Polar extracts of the brown alga *Fucus vesiculosus* (L.) reduce assimilation efficiency but do not deter the herbivorous isopod Idotea baltica (Pallas). *Journal of Experimental Marine Biology and Ecology*, 317(2), 143–157.

Kantz, K. & Singleton, V. L. (1990). Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *American Journal of Enology and Viticulture*, 41(3), 223-228.

Kim, J. A., Lee, J. M., Shin, D. B., & Lee, N. H. (2004). The antioxidant activity and tyrosinase inhibitory activity of phlorotannins in *Ecklonia cava*. *Food Science and Biotechnology*, 13 (4), 476-480.

Kim, S. K., Rajapakse, N., & Shahidi, F. (2007). Production of bioactive chitosan oligosaccharides and their potential use as nutraceuticals. In C. Barrow & F. Shahidi (Eds.), *Marine Nutraceuticals and Functional Foods* (pp. 297–319). Boca Raton, FL: CRC Press/Taylor & Francis Group.

Koivikko, R., Loponen, J., Honkanen, T., & Jormalainen, V. (2005). Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *Journal of Chemical Ecology*, 31(1), 195–212.

Li, Y, Qian, Z. J., Ryu, B. M., Lee, S. H., Kim, M. M., & Kim, S. K. (2009). Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*. *Bioorganic & Medicinal Chemistry*, 17(5), 1963–1973.

Mariod, A. A., Ibrahim, R. M., Ismail, M., & Ismail, N. (2009). Antioxidant activity and phenolic content of phenolic rich fractions obtained from black cumin (*Nigella sativa*) seedcake. *Food Chemistry*, 116(1), 306–312.

Miyashita, K., & Hosokawa, M. (2008). Beneficial health effects of seaweed carotenoid, fucoxanthin. In C. Barrow & F. Shahidi (Eds.), *Marine Nutraceuticals and Functional Foods* (pp. 297–319). Boca Raton, FL: CRC Press/Taylor & Francis Group.

Nakai, M., Kageyama, N., Nakahara, K., & Miki, W. (2006). Phlorotannins as radical scavengers from the extract of *Sargassum ringgoldianum*. *Marine Biotechnology*, 8(4), 409-414.

Oberholster, A. (2008). Investigation of the chemical and sensory properties of red wine pigments. University of Adelaide. Ph.D. Thesis. p. 66.

Parejo, I., Viladomat, F., Bastida, J., Rosas-Romero, A., Flerlage, N., Burillo, J. et al. (2002), Comparison between the radical scavenging activity and antioxidant activity of six distilled and nondistilled mediterranean herbs and aromatic plants. *Journal of Agricultural and Food Chemistry*, 50(23), 6882–6890.

Pavia, H. & Toth, G. B. (2000). Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus* v*esiculosus*. *Hydrobiologia*, 440(1-3), 299–305.

Plaza, M., Cifuentes, A., & Ibanez, E. (2008). In the search of new functional food ingredients from algae. *Trends in Food Science & Technology*, 19(1), 31-39.

Ragan, M. A., & Glombitza, K. W. (1986). Phlorotannins, brown algal polyphenols. In F. E., Round, & D. J., Chapman (Eds.), *Progress in Phycological Research* (pp. 129-241). Bristol, UK: Biopress Ltd.

Raghavan, S., Kristinsson, H. G., & Leeuwenburgh, C. (2008). Radical scavenging and reducing ability of tilapia (*Oreochromis niloticus*) protein hydrolysates. *Journal of Agricultural and Food Chemistry*, 56(21), 10359-10367.

Rupérez, P., Ahrazem, O., & Leal, J. A. (2002). Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *Journal of Agricultural and Food Chemistry*, 50(4), 840–845.

Sánchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 76(2), 270–276.

Senevirathne, M., Kim, S. H., Siriwardhana, N., Ha, J. H., Lee, K. W., & Jeon, Y. J. (2006). Antioxidant potential of *Ecklonia cava* on reactive oxygen species scavenging, metal chelating, reducing power and lipid peroxidation inhibition. *Food Science and Technology International*, 12(1), 27-38.

Shahidi, F., & Naczk, M. (2004). Nutritional and pharmacological effects of food phenolics. In F. Shahidi, & M., Naczk (Ed.), *Phenolics in food and nutraceuticals*, (pp. 331–402). Boca Raton, FL, USA: CRC Press.

Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H., & Hama, Y. (2008). Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *Journal of Applied Phycology*, 20(5), 705–711.

Shin, H. C., Hwang, H. J., Kang, K. J., & Lee, B. H. (2006). An antioxidative and antiinflammatory agent for potential treatment of osteoarthritis from *Ecklonia cava*, Archives of pharmacal research, 29(2), 165–171.

Swanson, A. K. & Druehl, L. D. (2002). Induction, exudation and the UV protective role of kelp phlorotannins. *Aquatic Botany*, 73(3), 241–253.

Targett, N. M., & Arnold, T. M. (1998). Minireview-Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *Journal of Phycology*, 34(2), 195-205.

Turkmen, N., Sari, F., & Velioglu, Y. S. (2005). The effect of cooking methods on total phenolics and antioxidant activity of selected green vegetables. *Food Chemistry*, 93(4), 713–718.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic Seaweeds. *Food Chemistry*, 116(1), 240-248.

Yuan, Y. V., Westcott, N. D., Hu, C., & Kitts, D. D. (2009). Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. *Food Chemistry*, 112(2), 321-328.

Zaragoza, M. C., Lopez, D., Saiz, M. P., Poquet, M., Perez, J. Puig-Parellada P., et al. (2008). Toxicity and antioxidant activity in vitro and in vivo of two *Fucus vesiculosus* extracts. *Journal of Agricultural and Food Chemistry*, 56(17), 7773–7780.

Zhang, Q., Zhang, J. Z., Shen, J. K., Silva, A., Denis, D. A., & Barrow, C. J. (2006). A simple 96-well microplate method for estimation of total polyphenol content in seaweeds. *Journal of Applied Phycology*, 18(3–5), 445-450.

Figures Captions

Figure 1. Flow diagram for extraction and fractionation of phlorotannins from *F. vesiculosus*

Figure 2. Effect of ultra-filtered subfractions on PMA stimulated chemiluminescent emission of freshly prepared human mono-nuclear cells. The concentration of cells used for this assay was 5 x 10^5 cells/ mL. The reaction was started using 5 units of HRP. The samples were added at a final concentration of 2.4 μg/ mL of reaction mixture. See the method section for more details. ROS scavenging activity of the samples was measured by their ability to reduce the area under the chemiluminescence curve.

Figure 3. Effect of Sephadex subfractions on PMA stimulated chemiluminescent emission from human mono-nuclear cells. The concentration of cells used for this assay was 5 x 10^5 cells/ mL. The reaction was started using 5 units of HRP. Subfractions LH–2 to LH–6 were added at a final concentration of 1.5 μg/ mL of reaction mixture, and LH–1 was tested at 3.0 μg/ mL concentration level. See the method section for more details. ROS scavenging activity of the samples was measured by their ability to reduce the area under the chemiluminescence curve.

Table 1 Total extractable substances (TES), total phlorotannin content (TPC) and DPPH EC_{50} (μg/mL) of different extracts from *F. vesiculosus*

Extractant	TES[1)] (g /100 g)	TPC[2)] (g PGE/100 g)	DPPH[3)] EC_{50} (μg/mL)
Water (20°C)	24.4 ± 0.6^{b}	26.3 ± 0.2^{e}	11.61 ± 0.59^{b}
Water (70°C)	37.8 ± 0.5^{a}	18.0 ± 0.3^{f}	24.04 ± 0.70^{a}
80% MeOH	22.9 ± 0.4^{b}	37.4 ± 0.6^{b}	7.54 ± 0.36^{d}
80% EtOAc	12.0 ± 0.3^{d}	36.5 ± 0.7^{bc}	8.28 ± 0.39^{cd}
80% EtOH	21.3 ± 0.5^{c}	35.4 ± 0.2^{c}	7.92 ± 0.11^{cd}
50% EtOH	23.1 ± 0.3^{b}	32.2 ± 0.3^{d}	8.76 ± 0.29^{c}
70% acetone	20.2 ± 0.9^{c}	39.3 ± 0.5^{a}	7.32 ± 0.17^{d}

Each value is expressed as means ± S.D. (n = 3).

Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).

[1)] TES, g dried extract/100 g dried algal powder.

[2)] TPC, g phloroglucinol equivalents (PGE) /100g extract.

[3)] EC_{50}, concentration of extract (μg/mL) required to scavenge 50% of the DPPH• in the reaction mixture.

Table 2 DPPH radical scavenging activity of different solvent fractions of *F. vesiculosus* compared with standard antioxidants

Fractions	Inhibition percentage (%)					EC_{50} (μg/mL)
	2 (μg/mL)	5 (μg/mL)	10 (μg/mL)	16 (μg/mL)	20 (μg/mL)	
n-Hexane	4.6 ± 0.3^{f}	11.5 ± 1.0^{e}	21.8 ± 0.6^{f}	33.4 ± 0.4^{f}	39.1 ± 1.4^{d}	30.12 ± 2.90^{a}
Ethyl acetate	36.1 ± 1.2^{b}	64.0 ± 0.5^{b}	84.4 ± 0.2^{bc}	94.2 ± 0.5^{bc}	95.3 ± 0.7^{ab}	3.76 ± 0.22^{c}
n-butanol	25.3 ± 0.8^{d}	52.7 ± 0.8^{c}	79.7 ± 1.4^{d}	89.7 ± 0.7^{d}	92.6 ± 0.6^{b}	4.77 ± 0.25^{c}
Aqueous residue	5.2 ± 0.3^{f}	12.6 ± 0.9^{e}	23.9 ± 1.0^{e}	36.0 ± 0.8^{e}	43.9 ± 1.9^{c}	23.92 ± 0.89^{b}
BHT	31.0 ± 0.4^{c}	63.6 ± 0.2^{b}	85.4 ± 0.1^{b}	93.2 ± 0.3^{c}	95.4 ± 0.3^{a}	3.28 ± 0.09^{c}
α-tocopherol	15.3 ± 0.3^{e}	42.2 ± 0.6^{d}	82.4 ± 0.4^{c}	95.2 ± 0.3^{b}	95.7 ± 0.2^{a}	5.93 ± 0.04^{c}
L-ascorbic acid	38.0 ± 0.7^{a}	95.0 ± 0.3^{a}	96.8 ± 0.2^{a}	97.3 ± 0.4^{a}	97.6 ± 0.6^{a}	2.49 ± 0.06^{c}

Each value is expressed as means ± S.D. (n = 3).

Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Table 3 Relative yields, total phlorotannin content (TPC) and antioxidant activities of different Sephadex subfractions from EtOAc fraction of *F. vesiculosus*

Subfractions	Relative yields (%) [2)]	TPC (g PGE/100 g)	DPPH EC_{50} (μg/ml)	Fe^{2+} chelating ability (%)	Reducing power (mg ASE/g) [3)]
LH–1	7.3 ± 1.3^{d}	82.7 ± 1.5^{d}	4.23 ± 0.17^{a}	46.5 ± 1.4^{a}	760.3 ± 63.9^{a}
LH–2	7.5 ± 0.5^{d}	96.6 ± 0.9^{a}	2.79 ± 0.05^{d}	28.5 ± 0.4^{e}	790.3 ± 61.3^{a}
LH–3	7.4 ± 0.9^{d}	97.0 ± 0.7^{a}	2.82 ± 0.03^{d}	30.5 ± 0.8^{de}	822.3 ± 29.2^{a}
LH–4	12.7 ± 0.3^{c}	85.0 ± 0.9^{cd}	3.89 ± 0.07^{b}	32.3 ± 1.1^{d}	717.8 ± 56.4^{a}
LH–5	46.2 ± 2.4^{a}	91.3 ± 2.4^{b}	3.50 ± 0.13^{c}	38.0 ± 0.5^{c}	785.8 ± 77.1^{a}
LH–6	18.9 ± 2.0^{b}	87.5 ± 1.2^{bc}	3.71 ± 0.07^{bc}	41.9 ± 1.8^{b}	780.5 ± 82.1^{a}
Total % recovery [1)]	75.4 ± 1.3				

Each value is expressed as means ± S.D. (n = 3).

Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).

[1)] Total % recovery from 300 mg of EtOAc fraction loaded on the Sephadex LH–20 column.

[2)] Relative yields are expressed as (%) weight of individual freeze-dried subfraction.

[3)] ASE, ascorbic acid equivalents.

Table 4 Relative yields, total phlorotannin content (TPC) and antioxidant activities of different MW subfractions from EtOAc fraction of *F. vesiculosus*

Subfractions	Relative yields (%)	TPC (g PGE/100 g)	DPPH EC_{50} (μg/mL)	Fe^{2+} chelating ability (%)	Reducing power (mg ASE/g)
> 100 kDa	73.9 ± 1.6[a]	87.8 ± 2.3[b]	3.80 ± 0.13[a]	45.1 ± 1.0[a]	881.3 ± 40.2[a]
100–30 kDa	9.6 ± 0.6[b]	95.4 ± 1.3[a]	3.58 ± 0.07[a]	47.6 ± 1.3[a]	899.0 ± 38.0[a]
30–10 kDa	3.3 ± 0.3[c]	97.0 ± 1.5[a]	3.07 ± 0.04[b]	36.6 ± 1.8[b]	910.7 ± 27.5[a]
10–5 kDa	2.7 ± 0.2[c]	94.8 ± 0.5[a]	2.91 ± 0.09[b]	33.7 ± 0.6[b]	822.0 ± 40.3[ab]
< 5 kDa	10.4 ± 0.9[b]	83.1 ± 1.8[b]	3.62 ± 0.11[a]	25.1 ± 0.2[c]	734.0 ± 43.0[b]

Each value is expressed as means ± S.D. (n = 3).

Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Figure 1.

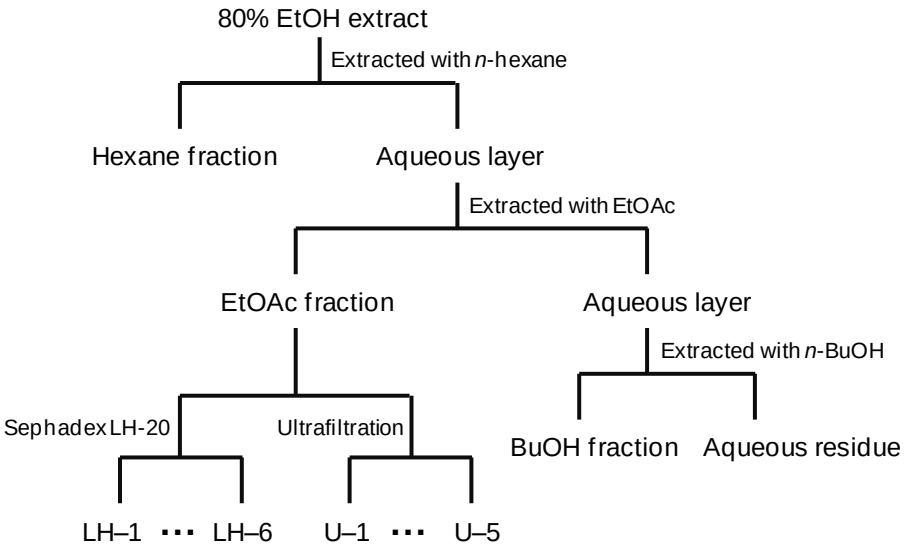

80% EtOH extract
Extracted with *n*-hexane
Hexane fraction
Aqueous layer
Extracted with EtOAc
EtOAc fraction
Aqueous layer
Extracted with *n*-BuOH
Sephadex LH-20
Ultrafiltration
BuOH fraction
Aqueous residue
LH–1 ··· LH–6
U–1 ··· U–5

Figure 2.

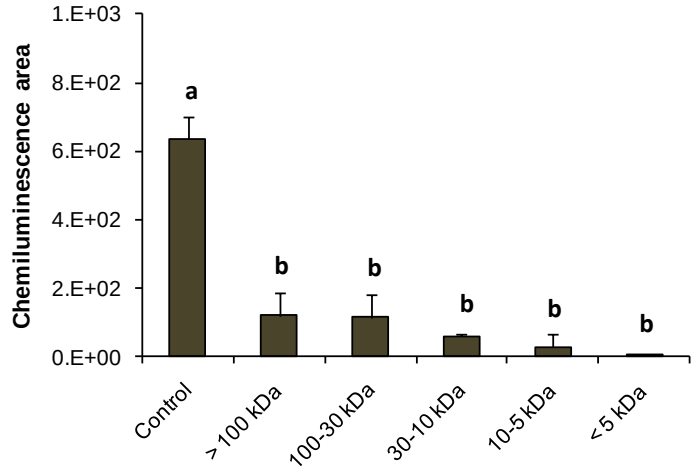
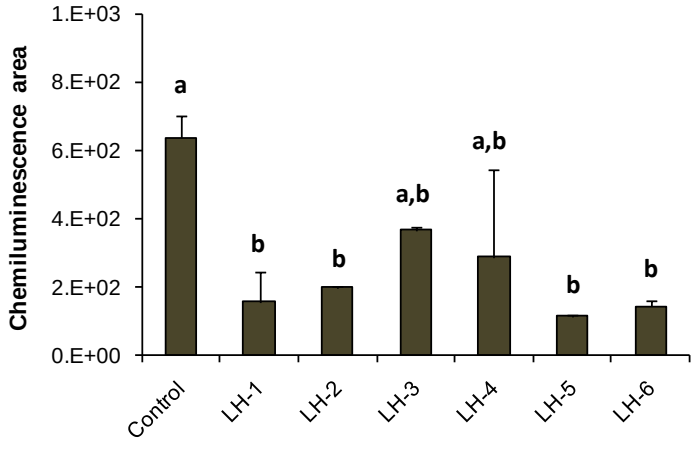


Figure 3.



PAPER V

Inhibition of Haemoglobin-Mediated Lipid Oxidation in Washed Cod Muscle and Cod Protein Isolates by *Fucus vesiculosus* Extract and Fractions

Tao Wang, Rósa Jónsdóttir, Hörður G. Kristinsson, Guðjón Þorkelsson, Charlotte Jacobsen, Patricia Yuca Hamaguchi, Gudrún Ólafsdóttir

V

Submitted for publication

Inhibition of haemoglobin-mediated lipid oxidation in washed cod muscle and cod protein isolates by *Fucus vesiculosus* extract and fractions

Tao Wang [1,2,3,*], Rósa Jónsdóttir [2], Hordur G. Kristinsson [2,4], Gudjon Thorkelsson[1,2], Charlotte Jacobsen [5], Patricia Yuca Hamaguchi [2] and Gudrún Ólafsdóttir [6]

[1]Faculty of Food Science and Nutrition, School of Health Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

[2]Matís ohf., Skulagata 4, 101 Reykjavik, Iceland

[3]Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

[4]Department of Food Science and Human Nutrition, University of Florida, Gainesville, FL.USA

[5]National Institute of Aquatic Resources, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

[6]School of Engineering and Natural Sciences, University of Iceland, Hjardarhagi 2-6, 107 Reykjavik, Iceland

*Corresponding Author

Faculty of Food Science and Engineering, Dalian Fisheries University, Heishijiao 52, 116023 Dalian, China

Tel: +86-411-84763557

Fax: +86-411-84763508

Email: wang_tao633@yahoo.com

Abstract

The effects of *F. vesiculosus* extract and fractions towards haemoglobin- (Hb-) catalysed lipid oxidation in washed cod muscle system and cod protein isolates during ice storage were examined. The extract and fractions were characterised in terms of total phlorotannin content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, ferrous ion-chelating ability and reducing power. Progression of oxidation was followed by determining rancid odour, thiobarbituric acid reactive substances (TBARS), redness and volatile oxidation compounds by gas chromatography (GC). In both washed cod muscle and protein isolates, phlorotannin-enriched ethyl acetate (EtOAc) fraction showed higher inhibitory effect than crude 80% ethanol (EtOH) extract. The addition of oligomeric phlorotannin-rich subfraction (LH-2) separated by Sephadex LH-20 chromatography, completely inhibited the initiation of lipid peroxidation in both systems throughout the entire study period (8 days). Its effectiveness at 300 mg/kg level was comparable to that of 100 mg/kg propyl gallate (PG), a highly effective synthetic antioxidant in muscle foods. Although polymeric phlorotannin-rich subfraction (LH-5) had similar level of TPC and chemical antioxidant activities as oligomeric subfraction LH-2, it was far less efficient in model systems. These results suggest that other factors rather than the intrinsic reactivity toward radicals could be responsible for the inhibitory effect of phlorotannins on lipid oxidation in fish muscle. This study highlights the great potential of oligomeric phlorotannins as novel natural antioxidants in fish and fish products.

Keywords: *Fucus vesiculosus*; phlorotannins; antioxidant; lipid oxidation; haemoglobin; washed cod muscle; cod protein isolates

1. Introduction

In recent years, there has been a growing interest in the use of natural phenolic antioxidants to enhance the oxidative stability, maintain or improve the intrinsic quality and nutritional value of seafood products (Lee, Krueger, Reed, & Richards, 2006a; Pazos, Gallardo, Torres, & Medina, 2005; Sanchez-Alonso, Borderias, Larsson, & Undeland, 2007). The antioxidant effects of various plant extracts as well as individual phenolic compounds have been evaluated and tested in different fish model systems. Green tea and tea catechins were reported as excellent antioxidants toward lipid oxidation in a cooked ground mackerel model system (He & Shahidi, 1997). The antioxidant effects of tea catechins were comparable to or even better than those of synthetic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and *tert*-butylhydroxyquinone (TBHQ) and the natural antioxidant α-tocopherol. A flavonol-enriched fraction produced from cranberry juice powder and white grape dietary fibre concentrate were also found to provide excellent protection against Hb-induced lipid oxidation in washed cod muscle model (Lee et al., 2006a; Sanchez-Alonso et al., 2007). Medina, Gallardo, Gonzalez, Lois, and Hedges (2007) studied the antioxidant potency of two different families of phenolic compounds, hydroxycinnamic acids and catechins in chilled minced horse mackerel muscle. Caffeic acid was found to be the most effective in preventing oxidative rancidity and the antioxidant potency was highly related to its ability to donate electrons.

Marine algae are emerging as a viable and plentiful source of natural antioxidants with remarkable and diverse claims for health-promoting properties. A number of potent antioxidant compounds have been isolated and identified from different types of seaweeds, including phlorotannins, sulphated polysaccharides, carotenoid pigments such as fucoxanthin and astaxanthin, sterols, catechins and mycosporine-like amino acids (MAAs) (Miyashita & Hosokawa, 2008; Rupérez, Ahrazem, & Leal, 2002; Yuan, Westcott, Hu, & Kitts, 2009). Algal polyphenols, especially phlorotannins (polyphloroglucinol phenolics) derived from brown algae have recently become a focus of intensive research due to their superior antioxidant properties. Several phlorotannin oligomers including eckol, phlorofucofuroeckol A, dieckol and 8,8′-bieckol were isolated from the Japanese Laminariaceous brown algae *Eisenia bicyclis*, *Ecklonia cava* and *Ecklonia kurome* and exhibited noteworthy antioxidant activities against DPPH and superoxide anion radicals, which were around 2 to 10 times more effective than catechin, α-tocopherol and ascorbic acid (Shibata, Ishimaru, Kawaguchi, Yoshikawa, & Hama, 2008). However, most of these studies have mainly focused on pharmaceutical and biomedical applications. There is lack of information about the antioxidant effects of algal polyphenols in food systems, especially muscle foods. Moreover, the antioxidant properties of some seaweed extracts or purified phlorotannin compounds have, so far, only been characterised using simple and fast *in vitro* tests. Many of these assays such as DPPH and ORAC monitor scavenging activity against a single target radical under certain reaction conditions which are not relevant to real food systems (Frankel, 2007; Frankel & Meyer, 2000). Therefore, these chemical antioxidant assays

cannot be expected to adequately predict the efficacy of algal polyphenols in complex muscle foods where multiple oxidation reactions may occur simultaneously and not only the intrinsic antioxidant property but also the physical location and orientation of antioxidant are important and decisive for the antioxidant effectiveness.

A well-designed model system comprised of washed fish muscle and added Hb as oxidation catalyst is being widely employed for oxidation study of fish muscle (Decker, Warner, Richards, & Shahidi, 2005; Richards & Hultin, 2000). It has intact myofibrillar proteins and membranes which closely resembles the physical structure of fish muscle. Compared with other model systems such as bulk oil, emulsions or microsomes, the performance of candidate antioxidant in washed fish model can provide more accurate predictions of its antioxidant effectiveness in real fish muscle system.

The production of protein isolates with improved stability and functionality from fish by-products and low value underutilised fish species are of great interest in the fish industry. Although different amount of membrane lipids can be removed during acid- and alkali-aided protein isolation, the residual membrane phospholipids still readily undergo oxidation in the presence of strong prooxidants like Hb, which limits their effective utilisation for surimi production and as injection or tumbling marinades to improve water holding capacity of fish fillets (Nolsøe & Undeland, 2009; Raghavan & Hultin, 2009). The use of seaweed derived antioxidants may offer a novel approach to enhance the oxidative stability of fish protein isolates.

Our recent screening study of ten species of common Icelandic seaweeds indicated that *F. vesiculosus* had the highest TPC, the greatest scavenging activities against DPPH and peroxyl radicals as well as a moderate ferrous ion-chelating ability (Wang, Jónsdóttir, & Ólafsdóttir, 2009). A parallel study showed that phlorotannins are the main contributors to the antioxidant activity of *F. vesiculosus*. Oligomeric and polymeric phlorotannin-rich fractions had similar DPPH scavenging activity, which was comparable to or even higher than the commercial antioxidants (unpublished data). The information about the antioxidant effect and specific mechanism of *F. vesiculosus* extract in fish model is greatly needed in order to justify its potential application to improve the oxidative stability of fish muscle and fish protein based products. Therefore, the objective of the present study was to evaluate the ability of *F. vesiculosus* extract and fractions to prevent Hb-mediated lipid oxidation in a washed cod model system and cod protein isolates, to evaluate the contribution of phlorotannins to the overall antioxidant effect of the crude extracts and to gain a basic understanding of how intrinsic antioxidant characteristics, molecular size may influence their antioxidant effectiveness.

2. Materials and methods

2.1. Materials

The brown algae (Phaeophyta) *Fucus vesiculosus* (Linnaeus) was collected in Hvassahraun coastal area nearby Hafnarfjordur, southwestern Iceland on October 15th, 2008. The seaweeds were washed

with clean seawater to remove epiphytes and sand attached to the surface and transported to the laboratory. The samples were carefully rinsed with tap water. Small pieces were cut and then freeze-dried, pulverised into fine powder and stored in tightly sealed polystyrene containers at −20 °C prior to extraction.

Fresh cod (*Gadus morhua*) fillets used for the preparation of washed cod model and protein isolates were obtained iced from Marland Ltd. (Reykjavík, Iceland) within 24-48 h from time of catch. Freshness was considered to be good to excellent based on odour and appearance. The fillets were skinned and all dark muscle, blood spots and excess connective tissue was removed. The white muscle was minced in a grinder (plate hole diameter 4.5 mm).

2.2. Chemicals

All the solvents used were of HPLC grade. All of the chemicals were of analytical grade and obtained from Sigma–Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) or Sigma–Aldrich (Steinheim, Germany).

2.3. Preparation of F. vesiculosus extract and fractions

The preparation procedures of *F. vesiculosus* extract and fractions were based on the parallel study (unpublished data). Briefly, forty grams of dried algal powder were extracted with 200 ml 80% EtOH in a platform shaker for 24 h at 200 rpm and at room temperature. The mixture was centrifuged at 2500 *g* for 10 min at 4 °C and filtered. The filtrate was concentrated *in vacuo* to a small volume and the residue was suspended in a mixture of methanol (MeOH) and water (40:30, v/v) and partitioned three times with *n*-hexane, EtOAc and *n*-butanol successively. The EtOAc soluble fraction, which exhibited the highest TPC, DPPH scavenging activity and reducing power (unpublished data), was subjected to gel filtration on a Sephadex LH-20 column and eluted stepwise with solvent systems of decreasing polarity, yielding six subfractions: LH-1 (50% aqueous MeOH, v/v), LH-2 (75% aqueous MeOH, v/v), LH-3 (pure MeOH), LH-4 (MeOH/acetone 5:1, v/v), LH-5 (MeOH/acetone 3:1, v/v) and LH-6 (MeOH/acetone 1:1, v/v). The solvent in each subfraction was evaporated to dryness under reduced pressure. The preparation was repeated three times. All the extract and fractions were lyophilised and stored in air-tight containers at -20 °C until further use.

Following extract and fractions were chosen for model studies: crude 80% EtOH extract, phlorotannin-enriched EtOAc fraction, Sephadex subfraction LH-2 which was rich in phlorotannin oligomers and LH-5 which was rich in phlorotannin polymers were used in washed cod model. Instead of the 80% EtOH extract, two more subfractions LH-3 and LH-6 were tested in cod protein isolates.

2.4. Partial characterisation of F. vesiculosus extract and fractions

2.4.1. Total phlorotannin content

The total phlorotannin content (TPC) of *F. vesiculosus* extract/fractions was determined by the Folin–Ciocalteu method described by Koivikko, Loponen, Honkanen, and Jormalainen (2005). Results were expressed as g phloroglucinol equivalents (PGE) per 100 g of extract.

2.4.2. DPPH radical scavenging activity

The DPPH radical scavenging activity was estimated according to the method of Sánchez-Moreno, Larrauri, and Saura-Calixto (1998). The EC_{50} value was calculated as the concentration of sample (μg/ml) required to scavenge 50% of the DPPH• in the reaction mixture.

2.4.3. Ferrous ion-chelating ability

The Ferrous ion-chelating ability was measured according to Decker and Welch (1990). All the extract/fractions were tested at a concentration of 5 mg/ml.

2.4.4. Reducing power

The reducing power was determined by the method of Benjakul, Visessanguan, Phongkanpai, and Tanaka (2005). Results were expressed as mg of ascorbic acid equivalents (ASE) per gram of sample.

2.4.5. Partitioning of F. vesiculosus fractions in fish oil/water system

The partitioning of *F. vesiculosus* fractions between fish oil and water was determined according to Lee, Reed, and Richards (2006b) and Pazos et al. (2005) with minor modifications. Briefly, 1ml of fish oil and 1 ml of distilled water containing *F. vesiculosus* fractions were vigorously vortexed twice for 30 s and centrifuged at 400 *g* for 20 min. The aqueous phase was transferred into an Eppendorf centrifuge tube and centrifuged again at 400 *g* for 20 min. The TPC in the aqueous phase before and after vortexing was measured. The partitioning of each fraction between fish oil and water was expressed as the percentage of PGE in the aqueous phase out of the total PGE before mixing.

2.5. Bleeding of fish, preparation of hemolysate and quantification of haemoglobin

Farmed Arctic char (*Salvelinus alpinus*) obtained from the Department of Aquaculture at Hólar Agricultural College (Hólar, Iceland) were anesthetised in phenoxy ethanol (0.5 g/l) for 3 min. The fish was held belly up and 1 ml of blood drawn from the caudal vein with a disposable syringe, preloaded with 1 ml of 150 mM NaCl and sodium heparin (30 units/ml).

Hemolysate was prepared within 24 h of blood collection according to the method of Richards et al. (2000). The heparinised blood was washed with four volumes of ice cold 1.7% NaCl in 1 mM Tris, pH 8.0. The plasma was removed by centrifugation at 700 *g* for 10 min at 4 °C. The red blood cells were then washed three times with ten volumes of the same buffer and centrifuged between washes as before. The cells were lysed in three volumes of 1 mM Tris, pH 8.0, for 1 h. The stroma was removed by adding one-tenth volume of 1 M NaCl before final ultracentrifugation at 28000 *g* for 15 min at 4 °C. All materials and samples were kept on ice during preparation. The hemolysate was stored at -80 °C until use.

The concentration of Hb was determined by the HemoCue system of plasma/low Hb microcuvettes and photometer (Hemocue, Ängelholm, Sweden), using a method based on Vanzetti's reagents and

spectrophotometric determination of azide-methaemoglobin complexes at 570 nm (Jónsdóttir, Bragadóttir, & Olafsdottir, 2007). A standard curve with serial bovine Hb solution (ranging from 0-70 μmol/l) was used for calibration. Samples and standards were diluted with 50 mM tris buffer (pH 8.6).

2.6. Washed cod muscle

Minced cod muscle was washed based on the method of Richards et al. (2000). All materials and samples were kept on ice during preparation. The mince was washed twice with Milli-Q water (1:3, w/w) and once with 50 mM sodium phosphate buffer (1:3, pH 6.3). The washed mince was immediately frozen and kept at -80 °C until used.

2.7. Preparation of cod protein isolates by alkaline solubilisation and isoelectric precipitation

Cod protein isolates were prepared as described by Kristinsson, Theodore, Demir, and Ingadottir (2005) with some modifications. The minced muscle tissue was homogenised in six volumes of cold Milli-Q water. The pH of the homogenate was adjusted to 10.8 to solubilise the protein and incubated at 4 °C for 30 min. The insoluble material was removed by filtration through cheesecloth. The pH of the homogenate was then adjusted to 5.5 to precipitate the myofibrillar protein. The precipitated protein was dewatered on a plastic sieve lined with cheesecloth and excess water was squeezed out manually. The final pH of protein isolates was adjusted to 7.0 and moisture content to 80%. Samples was immediately frozen and kept at -80 °C. Centrifugation step was eliminated from the protocol to maximise the amount of phospholipids available for oxidation.

2.8. Preparation of oxidation systems

The oxidation systems were prepared according to Richards et al. (2000) and Undeland, Kristinsson, and Hultin (2004). The washed cod model and the cod protein isolates were thawed under cold running tap water and adjusted to pH 6.3 with 1 M HCl and/or 1 M NaOH. Streptomycin sulphate (200 mg/kg) was added to the model to prevent microbial spoilage. Seaweed extract and fractions were added at a concentration of 300 mg/kg model based on preliminary studies (data not shown). Propyl gallate (PG) was at 100 mg/kg. Fifty percent aqueous EtOH (v/v) was used as carrier solvent for both seaweed samples and PG. The carrier comprised 1.0% of the final sample weight. The moisture content of all the samples was standardised to 85% for washed cod model and 81% for cod protein isolates with 50 mM sodium phosphate buffer (pH 6.3). An appropriate volume of hemolysate from Arctic char was added to a final concentration of 20 μmol Hb per kg mince to induce oxidation. In addition to the blank sample without Hb, a control sample containing the same concentration of EtOH without any added antioxidants was also included to minimise possible solvent effects.

For each sample group, approximately 25 g of the model was weighed and spread evenly at the bottom of 250 mL Erlenmeyer flasks (bottom diameter 80 mm) using an L-shaped stainless-steel spatula.

Each sample and blank was prepared in duplicate. The capped sample bottles were stored on ice inside a refrigerator (1-3 °C) for up to 8 days.

2.9. pH

The pH was measured with an Ag/AgCl combination electrode connected to a pH meter (Radiometer model PHM80, Radiometer, Copenhagen, Denmark). The pH of samples was determined after mixing 1 part of mince with 9 parts of Milli-Q water with a magnetic stirrer.

2.10. Measurement of redness (a)*

Changes in redness (*a**) of samples and blank were monitored during ice storage. The colour was measured by a Minolta CR-300 Chroma meter (Minolta Camera Co., Ltd., Osaka, Japan) as described by Jónsdóttir et al. (2007).

2.11. Thiobarbituric Reactive Substances (TBARS)

Samples were taken periodically according to the procedure described by Undeland et al. (2004). Sample plugs (~1 g) of the model were taken from the Erlenmeyer flasks with the aid of a plastic cylinder (diameter = 10 mm). The sample-plugs were transferred to Eppendorf centrifuge tubes and stored at -80 °C until measured. TBARS were determined according to direct extraction method (Sørensen & Jørgensen, 1996). TBARS values, expressed as µmol malondialdehyde per kg of sample (µmol MDA/kg), was calculated using a standard curve prepared from 1,1,3,3 tetraethoxypropane (TEP).

2.12. Sensory analysis

Four to five trained panelists evaluated the odour of the samples by sniffing the head space of the Erlenmeyer flasks. The panelists had several years of experience in evaluating rancidity of fish and fish oils.

Prior to the model study, two sessions were held for training to identify the rancid odour by using Hb treated cod muscle model samples with different storage time on ice. Typical overall rancid odour that develops is complex and can be described like dried fish like, oxidised cod liver oil and painty or grass like odour. The intensity of the overall rancid odour for a given sample was discussed under the guidance of the panel leader by using an unscaled line (100 mm long) from 0 to 100. 0-10 indicated not detectable, 10-20 borderline, 20-30 detectable, 30-40 moderate, 40-50 considerable, 50-100 dominant. Reference samples were prepared from almost odourless soybean oil containing 2.5% and 10% oxidised cod liver oil and used to establish a borderline and the point of considerable rancidity on the scale.

Sensory evaluation was conducted under standardised conditions. The testing area was illuminated with a red light bulb to mask subtle colour differences between samples. Capped samples were

allowed to rest for 45 min on ice between panelists' sessions, in order to recover equilibrium in the headspace of the samples.

2.13. Volatile compounds by GC

Volatile compounds were analysed in the control (Hb) group, 80% EtOH extract and EtOAc fraction in the washed cod model system after 0, 1, 3 and 4 days of storage and in control (Hb), LH-2 and LH-5 subfractions in cod protein isolates, after 0, 2 and 4 days of storage.

Sample of washed cod model or isolate model was weighed (4.5 – 5.0 g) into a 5 ml sealed vial for headspace Solid Phase Microextraction (HS-SPME) / Gas Chromatography analysis (GC-O and GC-FID). Heptanoic acid ethyl ester was added as an internal standard to the sample by adding 0.5 ml of 10 µl/l aqueous solution of the standard. Duplicate analyses of each sample were performed. Samples were kept at 25 °C for about 15 min before sample collection and the headspace volatiles collected for 40 min. Separation, identification and quantification of volatile compounds collected by the SPME technique was done by combined GC-O and GC-FID analysis as described earlier (Jónsdóttir et al., 2007). Gas Chromatography-Mass Spectrometry (GC-MS) was used to confirm the identity of volatiles but not for quantification. Prior to GC-MS, the sample (4.5 – 5.0 g) was dissolved in equal amount of saturated aqueous NaCl solution into a 250 ml round bottom flask. Heptanoic acid ethyl ester was added as an internal standard to all samples by adding 0.5 ml of 10 µl/l aqueous solution of the standard. The sample was purged at room temperature with nitrogen at about 100 ml/min for 2.5 h (15 l). Volatiles were collected on Tenax 60/80 for combined ATD 400 and GC-MS measurements as described earlier (Jónsdóttir et al., 2007).

2.14. Fatty acid profile of the washed cod muscle and cod protein isolates

The fatty acid composition of the washed cod muscle and cod protein isolates was determined by gas chromatography. Total lipid was extracted according to the method of Bligh and Dyer (1959). The methylation of fatty acids was carried out according to the AOCS (1998). The fatty acid methyl esters (FAME) were analysed using a Varian 3900 GC (Varian, Inc., Walnut Creek, CA, USA) equipped with a fused silica capillary column (HP-88, 100m X 0.25mm X 0.20 µm film, Agilent Technologies) and flame ionisation detector fitted with Galaxie Chromatography Data System, Version 1.9.3.2 software. The programme is based on AOAC (2000). Individual fatty acids were identified by comparison of their retention times with authentic standards. Results were expressed as percentages of the total fatty acid methyl esters.

2.15. Data analysis

The panelists' performance was analysed using PanelCheck V1.3.2 (www.matforsk.no/panelcheck). At each sampling, the samples were presented as averages over panelists' scores. To illustrate sample variation, the range between maximum and minimum values was used.

All other experiments were replicated two to three times (n = 2 – 3), and analyses were performed between two and six times (a = 2 – 6). Analysis of variance (ANOVA) was applied to the data using the Number Cruncher Statistical Software, NCSS 2000 (NCSS, Kaysville, Utah, USA). Significant differences were determined by one way ANOVA and Tukey-Kramer Multiple-Comparison Test was used to determine the statistical difference between sample groups. Significance of differences was defined at the 5% level ($p<0.05$).

3. Results

3.1. Partial characterisation of F. vesiculosus extract and fractions

The *F. vesiculosus* crude extract and fractions used in the model studies were assessed for TPC, DPPH radical scavenging activity, ferrous ion-chelating ability and reducing power. The partitioning behaviour of Sephadex subfractions was studied in fish oil/water system.

The crude 80% ethanol extract had a TPC value of 35.4 g PGE/ 100 g extract (Table 1). After solvent partitioning, most of the phlorotannin constituents were effectively enriched in the EtOAc fraction (88.3 g PGE/ 100 g extract). Among the four Sephadex subfractions, LH-2 and LH-3 contained the highest level of TPC while the lowest amount was found in LH-6. Consistent with the TPC assay, EtOAc fraction was 2.1 times more effective at scavenging DPPH radicals than the crude extract. Subfraction LH-2 (EC_{50} = 2.79 μg/ml) and LH-3 (EC_{50} = 2.82 μg/ml) showed significantly higher DPPH scavenging activity than the other subfractions and the original EtOAc fraction. The activity was comparable to those of the positive controls, L-ascorbic acid (EC_{50} = 2.5 μg/ml) and BHT (EC_{50} = 3.3 μg/ml), and was significantly higher than that of α-tocopherol (EC_{50} = 5.9 μg/ml). Similar trends were observed with reducing power. With regard to ferrous ion-chelating ability, all the *F. vesiculosus* extract and fractions (at 5 mg/ml) exhibited higher capacity than those of L-ascorbic acid (8.2% at 20 mg/ml) and citric acid (21.5% at 5 mg/ml), but was much lower than that of the potent metal chelator, EDTA-Na_2 (99.8% at 0.05 mg/ml).

Sephadex LH-20 gel has been widely used for the fractionation and purification of different groups of phenolic compounds from various plant and algal materials. By using appropriate eluent system, Sephadex LH-20 column can also be employed to separate non-polymeric and polymeric phenols (Kantz & Singleton, 1990; Shahidi & Naczk, 2004**)**. Based on the separation principle and chromatographic behaviour of phlorotannin compounds in different subfractions by TLC (unpublished data), LH-2 and LH-3 were composed mainly of phlorotannin oligomers while LH-5 and LH-6 contained primarily polymeric phlorotannins.

As shown in Table 2, the polarity of different *F. vesiculosus* subfractions decreased in the order of LH-3 > LH-2 > LH-5 > LH-6. Subfractions LH-2 and LH-3 were highly polar and most of them were retained within the aqueous phase after the mixing. LH-5 was less polar than LH-2 and LH-3. LH-6

was more hydrophobic than any other fraction and was distributed almost equally between the oily and aqueous phase.

3.2. Comparison of F. vesiculosus extract and fractions on the inhibition of lipid oxidation in washed cod model

The addition of Hb induced the oxidation of the model as shown by rapid generation of intense rancid odour (odour score above 50) in control sample within two days of ice storage (Figure 1a). During the eight day storage period, the blank sample without Hb did not exhibit any detectable rancid odour. When Hb was added to the samples, a characteristic, initial odour was detected in all groups on day 1 (less than odour score 20). The addition of the crude 80% EtOH extract prolonged the lag phase for initial odour detected on day 1 to about 3 days while samples treated with phlorotannin-enriched EtOAc fraction led to an extension of the lag phase by about one more day. The higher oxidative stability effect of EtOAc fraction could be ascribed to its higher TPC level. The Sephadex subfraction LH-2 was very effective in preventing lipid oxidation and no rancid odour was detected during the entire study period. Its effectiveness at 300 mg/kg level was comparable to that of 100 mg/kg PG, which is known as one of the most effective antioxidants in muscle food systems. On the contrary, subfraction LH-5 showed only a slight retarding effect towards rancidity development and provided about one day of oxidative shelf-life extension over the untreated control.

In accordance with the sensory data, the development of TBARS was fast in the control sample which contained no antioxidants and reached a maximum level at day 2 of ice storage (Figure 1b). There was a prolonged lag time for the formation of TBARS in samples treated with *F. vesiculosus* extract and fractions. The EtOAc fraction showed a greater inhibitory effect than the crude 80% EtOH extract. Incorporation of subfraction LH-2 at 300 mg/kg and PG at 100 mg/kg completely inhibited TBARS formation throughout the whole storage period. Consistent with sensory results, subfraction LH-5 exerted only a weak inhibition of TBARS formation.

Volatile compounds identified by GC-MS and GC-O/GC-FID analysis after 4 days storage were mainly alcohols, aldehydes and ketones which have been associated with lipid oxidation (Table 3). The key volatiles detected responsible for rancid odour were the following: 1-penten-3-ol, 1-octen-3-ol (mushroom odour), hexanal (grass odour), *cis*-4-heptenal and heptanal (boiled potato/fishy odour) and 2,4-heptadienal (sweet, citrus like odour). Although none of the volatiles are characterised as rancid themselves, their combined effect contributes to the overall rancid odour (Jónsdóttir et al, 2007). The most rapid formation of volatile oxidation compounds was observed in the control sample which contained no antioxidants and the amount of key volatiles reached about 430 ng/g after 4 days storage (Figure 1c), hexanal being the most abundant volatile (240 ng/g). The development of volatiles was much slower in the groups containing the crude extract and EtOAc fraction.

Deoxygenated and/or met-Hb have previously been reported to be more active than other forms of haemeproteins in initiating lipid oxidation (Richards et al., 2000). The transformation of oxy-Hb into

met-Hb can be estimated instrumentally by the loss of red colour (Wetterskog & Undeland, 2004). As shown in Figure 1d, there was some variation in *a** value among sample groups on day 0, which was caused by the colour differences between various seaweed extract and fractions. Samples containing the EtOAc fraction and LH-2 had slight higher initial *a** value than the control group, while those treated with crude extract and LH-5 had lower *a** value. During ice storage of the samples, no significant change in redness was observed in blank sample without Hb. EtOAc fraction was more effective in retaining the red colour than the crude extract. The slowest rate of redness loss was observed in the cod model supplemented with LH-2. Although the addition of EtOAc fraction and LH-2 resulted in an increase in the redness and could exert slight masking effects on the potential transformation of Hb, the progress of redness loss correlated well with the sensory and TBARS data which demonstrated that change in *a** value is a quick and reliable indicator of the oxidation process in washed fish model system as suggested by Wetterskog et al. (2004).

3.3. Effect of EtOAc fraction and its subfractions on the oxidative stability of alkali-solubilised cod muscle protein isolates

Owing to the different antioxidant behaviour of subfractions LH-2 and LH-5 observed in washed cod model, it was therefore of particular interest to test and compare the ability of various Sephadex LH-20 subfractions to inhibit Hb-catalysed lipid oxidation in cod protein isolates. The two systems had equal fat content (0.5%) and also similar fatty acid composition (Table 4). However, the oxidation process of protein isolates was slower and less pronounced than the washed cod model. The antioxidant potency of EtOAc fraction and its subfractions in inhibiting the formation of rancid odour and TBARS was in the decreasing order of LH-2 ~ LH-3 > EtOAc fraction > LH-5 ~ LH-6 (Figure 2a and 2b). The development of TBARS was fast in the control sample and reached a maximum level of 40 µmol MDA/kg of sample after 3 days of ice storage. Subfractions LH-2 and LH-3 at a concentration of 300 mg/kg and PG at a concentration of 100 mg/kg exhibited a marked effect in suppressing the formation of TBARS, almost equally effective. Subfractions LH-5 and LH-6 were the least active and only provided one day of extension over the untreated control (Figure 2b).

The development of volatile oxidation compounds was in accordance with the sensory analysis and TBARS results. The fastest development was found in control sample (42.2 ng/g on day 4) and subfraction LH-2 almost totally suppressed the formation of the volatile oxidation compounds (7.9 ng/g on day 4). LH-5 (24.2 ng/g on day 4) was much less active than LH-2 (Figure 2c). According to the GC-FID results, c*is*-4-heptenal and heptanal and 2,4-heptadienal were in very low concentration or not detected at all but hexanal and 1-penten-3-ol were the most abundant volatile compounds in the fish isolate system.

The inhibition of lipid oxidation and rancid odour by LH-2, LH-3 and PG was also supported by the slow rate of redness loss (Figure 2d), although LH-2 was less effective in retarding loss of redness than in washed cod model system.

4. Discussion

Recent studies have indicated that oligomeric and polymeric phlorotannins are more potent antioxidants than the monomer phloroglucinol and oligomerisation of phloroglucinol appears to be crucial for the radical scavenging capacity of phlorotannins (Cerantola, Breton, Ar Gall, & Deslandes, 2006; Kim, Lee, Shin, & Lee, 2004). However, the relationship between molecular structure and antioxidant activity of phlorotannin compounds is still poorly understood. In a previous study, Cerantola et al. (2006) isolated two types of structurally distinct phlorotannin compounds from *Fucus spiralis* extracts. Both phlorotannin polymers showed equally high DPPH radical scavenging activity and no correlation was found between structural characteristics and the radical scavenging activity. Similarly, in our study, no clear trend in the chemical antioxidant activities was observed among oligomeric and polymeric phlorotannin-rich subfractions, but the difference in the reducing and DPPH scavenging activities appeared to be explained primarily by the difference in TPC. Phlorotannin oligomers and polymers in different subfractions did not seem to differ markedly in chemical antioxidant activity (Table 1 and unpublished data). However, several other studies have suggested that the degree of polymerisation or the number and arrangement of phenolic hydroxyl groups are important factors influencing the antioxidant effectiveness in oil model systems. High molecular weight fractions (containing phlorotannin polymers such as dieckol, phlorofucofuroeckol and 8-8′ bieckol) were more effective than low molecular weight fractions (containing phloroglucinol and eckol) in preventing rancidity of fish oil and autoxidation of methyl α-linolenate (Nakamura, Nagayama, Uchida, & Tanaka, 1996; Yan, Li, Zhou, & Fan, 1996). Therefore, the antioxidant effectiveness of phlorotannins appears to be system dependent and needs to be investigated further in relevant models which closely mimic the targeted food system.

It has been widely accepted that the main substrates of lipid oxidation in fish muscle tissue are phospholipids associated with cellular membranes. Directing antioxidants into muscle cell membranes has been assumed to enhance the oxidative stability of muscle tissue (Ballesteros, 2009; Kathirvel & Richards, 2009; Raghavan & Hultin, 2004). Several studies show that the partitioning behaviour of plant-derived polyphenols has a great impact on their ability to protect membrane lipids from deleterious oxidation in a variety of biological and food model systems (Arora, Byrem, Nair, & Strasburg, 2000; Caturla, Vera-Samper, Villalain, Mateo, & Micol, 2003). Galloylated catechins, in particular (–)-epicatechin gallate (ECG), were found to be more effective than their nongalloylated analogues in preventing lipid peroxidation in phospholipid model membranes (Caturla et al., 2003). The antioxidant potency was related to their ability to modulate the structure and function of membranes. Galloylated catechins were observed to penetrate deeper into the hydrophobic region of the membrane than their homologues, thus promoting the formation of ordered lipid domains and sterically hindering diffusion of free radicals through the membrane. Lee et al. (2006a) reported that a flavonol-rich fraction of intermediate polarity prepared from cranberry juice powder was highly

effective in preventing Hb-mediated lipid oxidation in washed cod muscle while proanthocyanidins with higher polarity and larger molecular size showed rather poor activity. The extraordinary effectiveness of flavonols was explained by its preferential location and orientation in the membrane bilayer which ensures effective scavenging of lipophilic alkoxyl and hemin radicals. Interestingly, in the present study, most of the phlorotannins in different Sephadex subfractions were more soluble in the water than in the oil phase (Table 2). A simple or direct relationship could not be established between the polarity of the subfractions and their antioxidant effectiveness in fish model systems, which reflects the complexity of mechanism involved in antioxidant protection in a heterogeneous fish matrix. Moreover, the highly efficient subfractions LH-2 and LH-3 were more polar than LH-5 and LH-6. Because of the hydrophilic nature of phlorotannin oligomers in LH-2 and LH-3, they are not likely to partition preferentially into the hydrophobic interior of the membrane. Instead, they might be localised in close proximity to the membrane surface to scavenge aqueous phase radicals. Therefore, the antioxidant potency of phenolic antioxidants in fish model systems may not necessarily depend on their ability to penetrate into the membrane. PG has been demonstrated to be a particularly effective inhibitor of lipid oxidation in fish systems in both the present and previous studies. It has recently been reported that PG had the highest water solubility and the least tendency to partition into the membrane phase of washed cod model among three gallic acid esters (propyl gallate, octyl gallate and lauryl gallate) (Ballesteros, 2009). The association of PG with the hydrophilic region of phospholipid bilayer was also confirmed by previous spectroscopic spectral shift and fluorescence quenching studies (Dwiecki, Gornas, Nogala-Kalucka, & Polewski, 2006). PG was therefore assumed to function mainly as an aqueous phase antioxidant (Ballesteros, 2009).

The exact mechanisms accounting for the differential antioxidant effects of these phlorotannin components in fish models are not clear. One possible explanation could be that the small size and high polarity of oligomeric phlorotannins present in fractions LH-2 and LH-3 might allow them to reside close to the polar surface of the membrane, effectively quenching radicals generated in the aqueous phase and converting them to more stable and non-radical compounds. Therefore, phlorotannin oligomers appear to act mainly as the first line of defense by quenching aqueous phase radicals before they attack the membranes, rather than as scavengers of chain-propagating lipid peroxyl radicals within the membrane. Since free radical attack from the aqueous phase is known to be an important factor initiating membrane lipid peroxidation, efficient quenching of aqueous peroxyl radicals near the membrane surface may contribute greatly to the retardation of the onset of lipid oxidation (Paiva-Martins, Gordon, & Gameiro, 2003). On the other hand, the bulky size of highly polymeric phlorotannins in fractions LH-5 and LH-6 might sterically hinder their interactions with the membrane bilayer, which could partly explain the lack of effect in both systems. In addition, these subfractions might also contain high molecular weight complexes formed between phlorotannins and other macromolecules like proteins or carbohydrates. The complexation may greatly impair the ability of phlorotannins to protect membrane phospholipids from oxidation. Several plant-derived

polyphenols such as catechins and olive oil phenols were shown to regenerate α-tocopherol effectively (Mukai, Mitani, Ohara, & Nagaoka, 2005; Paiva-Martins et al, 2003). Similarly, phlorotannin oligomers may also have the ability to regenerate the endogenous antioxidant α-tocopherol in fish muscle through a H-transfer mechanism. However, further investigations are needed to extend our findings and elucidate the precise mechanisms of the antioxidant effect of phlorotannins in fish muscles.

The analysis of volatile compounds further confirmed the effect of 80% EtOH extract and EtOAc fraction in the washed cod model and the superior efficiency of the LH-2 subfraction in the cod protein isolates (Figure 1c; Figure 2c). Hexanal that contributes to grass like odour, was the most abundant compound in the Hb treated control sample of washed cod model system together with 1-penten-3-ol and to less extent 1-octen-3-ol. Other key compounds derived from PUFA-3 with very low odour threshold were detected in lower levels but still influenced the overall odour. 80% EtOH extract and EtOAc fraction were effective in preventing the generation of volatile oxidation compounds and the sum concentration of the key volatiles were all significantly lower than the control sample (Figure 1c). In the control sample and LH-5 group of the protein isolates, the *n*-3 PUFA derived compounds c*is*-4-heptenal, heptanal and 2,4-heptadienal contributing to fishy odour, were in very low levels, but potential *n*-6 PUFA derived components like hexanal and 1-penten-3-ol giving green odour notes were the most abundant (data not shown). LH-2 appeared to be very effective in preventing the generation of volatile oxidation compounds. In particular, c*is*-4-heptenal, heptanal and 2,4-heptadienal were not detected at all (Table 3), indicating that oligomeric phlorotannins may be very useful to prevent formation of fishy odours in Hb induced oxidation of fish system. Further studies are needed to investigate also the efficiency of oligomeric phlorotannins towards other oxidative processes, e.g. lipoxygenase (LOX) activity in fish muscle. This is of importance to prevent the onset of oxidation in fish and generation of fishy odours (Fu, Xu, & Wang, 2009; Josephson, 1991).

When comparing the development of oxidation in the two systems, it is interesting to see that the intensity of oxidation is much lower in the isolates despite a similar fat content and fatty acid composition. The slower oxidation rate of cod protein isolates therefore can not be explained by the reduction of lipids after alkaline treatment or different amounts of susceptible polyunsaturated fatty acids. Previous papers have suggested that membrane phospholipids may become more buried and less accessible to pro-oxidants upon acid treatment followed by isoelectric precipitation (Kristinsson & Hultin 2004; Pazos, Medina, & Hultin, 2005; Vareltzis & Hultin, 2007). In this study the alkaline method was used and it is possible a similar conformational change of the membranes occurred at alkaline pH conditions, thus possibly explaining the difference between the isolate and washed muscle.

It was also of interest to examine if *in vitro* antioxidant tests could predict antioxidant effectiveness of phlorotannin antioxidants in complex fish systems. The results of the present study showed that the *in vitro* antioxidant activity did not consistently correlate with their relative ability to inhibit lipid oxidation in fish models. Subfraction LH-2, which had the second highest TPC and the strongest

DPPH radical scavenging activity, completely inhibited the initiation of lipid peroxidation in both washed cod muscle and alkali-solubilised cod protein isolates throughout the entire storage period. Interestingly, however, LH-5 with the third highest TPC level and considerable DPPH scavenging activity and reducing power, exerted only very weak inhibition in both model systems. The differential antioxidant effects observed herein reinforce the findings from early research that the ability of a phenolic compound to inhibit lipid oxidation in food systems can be influenced by a multitude of factors such as the intrinsic antioxidant property, the molecular size, location and orientation in targeted substrate where oxidation is prevalent as well as interactions with other food ingredients (Decker et al., 2005; Frankel, 2007; Kathirvel et al., 2009; Lee et al., 2006a).

5. Conclusions

The results of the present study showed that phlorotannins were the active components in *F. vesiculosus* extract responsible for the inhibition of lipid oxidation in fish model systems. Different phlorotannin components possessed variable antioxidant activity. Oligomeric phlorotannin-rich subfractions were highly efficient in suppressing the onset of lipid oxidation while polymeric phlorotannin-rich subfractions only had slight retardation effect. The effectiveness of subfraction LH-2 at 300 mg/kg level was comparable to that of 100 mg/kg PG, one of the most potent antioxidant in muscle food systems. *In vitro* antioxidant tests did not always reflect the antioxidant effectiveness of *F. vesiculosus* extract and fractions in fish model systems. The performance of phlorotannin antioxidants in heterogeneous fish muscle may not rely solely on the intrinsic reactivity toward radicals and partitioning into the membrane phase, but also on the antioxidant reactions occurring in the aqueous phase or other mechanisms that are not yet well understood.

Acknowledgements

This work was financed by AVS R&D Fund and Icelandic Research Fund and in collaboration with Íslensk hollusta ehf. The author Tao Wang would like to acknowledge the award of a PhD scholarship by United Nations University-Fisheries Training Programme. The authors would like to thank the sensory panel at Matís ohf.

References

AOAC (2000). AOAC official method 996.06, fat (total, saturated, and unsaturated) in foods. In *Official methods of analysis of AOAC international* (17th ed.). Gaithersburg, MD: AOAC International.

AOCS (1998). Official Method Ce 1b-89. In D. Firestone, *Official methods and recommended practices of the American Oil Chemists' Society* (5th ed.). Champaign, IL: American Oil Chemists' Society.

Arora, A., Byrem, T. M., Nair, M. G., & Strasburg, G. M. (2000). Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. *Archives of Biochemistry and Biophysics*, 373(1), 102-109.

Ballesteros, A. T. (2009). Antioxidant distribution and effectiveness in a model muscle system. University of Massachusetts Amherst. Ph.D. Thesis. pp. 30-57.

Benjakul, S., Visessanguan, W., Phongkanpai, V., & Tanaka, M. (2005). Antioxidative activity of caramelisation products and their preventive effect on lipid oxidation in fish mince. *Food Chemistry*. 90 (1-2), 231-239.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry* and *Physiology*, 37(8), 911-917.

Caturla, N., Vera-Samper, E., Villalain, J., Mateo, C. R., & Micol, V. (2003). The relationship between the antioxidant and the antibacterial properties of galloylated catechins and the structure of phospholipid model membranes. *Free Radical Biology and Medicine*, 34(6), 648-662.

Cerantola, S., Breton, F., Ar Gall, E., & Deslandes, E. (2006). Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. *Botanica Marina*, 49(4), 347-351.

Decker, E. A., Warner, K., Richards, M. P., & Shahidi, F. (2005). Measuring antioxidant effecticeness in food. *Journal of Agricultural and Food Chemistry*, 53(10), 4303-4310.

Decker, E. A., & Welch, B. (1990). Role of ferritin as a lipid oxidation catalyst in muscle food. *Journal of Agricultural and Food Chemistry*, 38(3), 674-677.

Dwiecki, K., Gornas, P., Nogala-Kalucka, M., & Polewski, K. (2006). Spectral properties of propyl gallate in organic solvents and in heterogeneous environment. *Acta Agrophysica*, 7(1), 39-48.

Frankel, E. N. (2007). *Antioxidants in Food and Biology: Facts and Fiction*. Bridgewater, England: The Oily Press.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *Journal of the Science of Food and Agriculture*, 80(13), 1925-1941.

Fu, X. J., Xu, S. Y., & Wang, Z. (2009). Kinetics of lipid oxidation and off-odor formation in silver carp mince: The effect of lipoxygenase and haemoglobin. *Food Research International*, 42(1), 85-90.

He, Y. H., & Shahidi, F. (1997). Antioxidant activity of green tea and its catechins in a fish meat model system. *Journal of Agricultural and Food Chemistry*, 45(11), 4262-4266.

Jónsdóttir, R., Bragadóttir, M., & Ólafsdóttir, G. (2007). The role of volatile compounds in odor development during hemoglobin-mediated oxidation of cod muscle membrane lipids. *Journal of Aquatic Food Product Technology*, 16(4), 67-86.

Josephson, D. B. (1991). Seafood. In H. Maarse (Eds.), *Volatile compounds in foods and beverages* (pp. 179-202). New York: Dekker.

Kantz, K., & Singleton, V. L. (1990). Isolation and determination of polymeric polyphenols using Sephadex LH-20 and analysis of grape tissue extracts. *American Journal of Enology and Viticulture*, 41(3), 223-228.

Kathirvel, P., & Richards, M. P. (2009). Mechanisms by which flavonol aglycones inhibit lipid oxidation better than glycosylated flavonols in comminuted muscle tissue. *Food Chemistry*, 117(1), 75-82.

Kim, J. A., Lee, J. M., Shin, D. B., & Lee, N. H. (2004). The antioxidant activity and tyrosinase inhibitory activity of phlorotannins in *Ecklonia cava*. *Food Science and Biotechnology*, 13(4), 476-480.

Koivikko, R., Loponen, J., Honkanen, T., & Jormalainen, V. (2005). Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *Journal of Chemical Ecology*, 31(1), 195-212.

Kristinsson, H. G., & Hultin, H. O. (2004). The effect of acid and alkali unfolding and subsequent refolding on the pro-oxidative activity of trout hemoglobin. *Journal of Agricultural and Food Chemistry*, 52(17), 5482-5490.

Kristinsson, H. G., Theodore, A. E., Demir, N., & Ingadottir, B. (2005). A comparative study between acid- and alkali-aided processing and surimi processing for the recovery of proteins from channel catfish muscle. *Journal of Food Science*, 70(4), C298-306.

Lee, C. H., Krueger, C. G., Reed, J. D., & Richards, M. P. (2006a). Inhibition of hemoglobin-mediated lipid oxidation in washed fish muscle by cranberry components. *Food Chemistry*, 99(3), 591-599.

Lee, C. H., Reed, J. D., & Richards, M. P. (2006b). Ability of various polyphenolic classes from cranberry to inhibit lipid oxidation in mechanically separated turkey and cooked ground pork, *Journal of Muscle Foods*, 17(3), 248-266.

Medina, I., Gallardo, J. M., Gonzalez, M. J., Lois, S., & Hedges, N. (2007). Effect of molecular structure of phenolic families as hydroxycinnamic acids and catechins on their antioxidant effectiveness in minced fish muscle. *Journal of Agricultural and Food Chemistry*, 55(10), 3889-3895.

Miyashita, K., & Hosokawa, M. (2008). Beneficial health effects of seaweed carotenoid, fucoxanthin. In C. Barrow & F. Shahidi (Eds.), *Marine Nutraceuticals and Functional Foods* (pp. 297-319). Boca Raton, FL: CRC Press/Taylor & Francis Group.

Mukai, K., Mitani, S., Ohara, K., & Nagaoka, S. I. (2005). Structure-activity relationship of the tocopherol-regeneration reaction by catechins. *Free Radical Biology and Medicine*, 38(9), 1243-1256.

Nakamura, T., Nagayama, K., Uchida, K., & Tanaka, R. (1996). Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fisheries Science*, 62(6)**,** 923-926.

Nolsøe, H., & Undeland, I. (2009). The acid and alkaline solubilisation process for the isolation of muscle proteins: state of the art. *Food and Bioprocess Technology*, 2(1), 1-27.

Paiva-Martins, F., Gordon, M. H., & Gameiro, P. (2003). Activity and location of olive oil phenolic antioxidants in liposomes. *Chemistry and Physics of Lipids*, 124(1), 23-36.

Pazos, M., Gallardo, J. M., Torres, J. L., & Medina, I. (2005). Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chemistry*, 92(3), 547-557.

Pazos, M., Medina, I., Hultin, H. O. (2005). Effect of pH on hemoglobin-catalyzed lipid oxidation in cod muscle membranes in vitro and in situ. *Journal of Agricultural and Food Chemistry*, 53(9), 3605-3612.

Raghavan, S., & Hultin, H. O. (2004). Distribution of exogenous delta-tocopherol between the membrane lipids and triacylglycerols of a cod muscle-triacylglycerol model system. *Journal of Agricultural and Food Chemistry*, 52(20), 6294-6299.

Raghavan, S., & Hultin, H. O. (2009). Effects of various antioxidants on the oxidative stability of acid and alkali solubilized muscle protein isolates. *Journal of Food Biochemistry*, 33(2), 163-175.

Richards, M. P., & Hultin, H. O. (2000). Effect of pH on lipid oxidation using trout hemolysate as a catalyst: a possible role for deoxyhemoglobin. *Journal of Agricultural and Food Chemistry*, 48(8), 3141-3147.

Rupérez, P., Ahrazem, O., & Leal, J. A. (2002). Potential antioxidant capacity of sulfated polysaccharides from the edible marine brown seaweed *Fucus vesiculosus*. *Journal of Agricultural and Food Chemistry*, 50(4), 840-845.

Sanchez-Alonso, I., Borderias, J., Larsson, K, & Undeland, I. (2007). Inhibition of hemoglobin-mediated oxidation of regular and lipid-fortified washed cod mince by a white grape dietary fiber. *Journal of Agricultural and Food Chemistry*, 55(13), 5299-5305.

Sanchez-Moreno, C., Larrauri, J. A., & Saura-Calixto, F. (1998). A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 76(2), 270-276.

Shahidi, F., & Naczk, M. (2004). Nutritional and pharmacological effects of food phenolics. In F. Shahidi, & M., Naczk (Ed.), *Phenolics in food and nutraceuticals*, (pp. 331–402). Boca Raton, FL, USA: CRC Press.

Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H., & Hama, Y. (2008). Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *Journal of Applied Phycology*, 20(5), 705-711.

Sørensen, G., & Jørgensen, S. S. (1996). A critical examination of some experimental variables in the 2-thiobarbituric acid (TBA) test for lipid oxidation in meat products. *Z Lebensm Unter Forsch*, 202(3), 205-210.

Undeland, I., Kristinsson, H. G., & Hultin, H. O. (2004). Hemoglobin-mediated oxidation of washed minced cod muscle phospholipids: Effect of pH and hemoglobin source. *Journal of Agricultural and Food Chemistry*, 52(14), 4444-4451.

Vareltzis, P., & Hultin, H. O. (2007). Effect of low pH on the susceptibility of isolated cod (*Gadus morhua*) microsomes to lipid oxidation. *Journal of Agricultural and Food Chemistry*, 55(24), 9859-9867.

Wang, T., Jónsdóttir, R., & Ólafsdóttir, G. (2009). Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic Seaweeds. *Food Chemistry*, 116(1), 240-248.

Wetterskog, D., & Undeland, I. (2004). Loss of redness (*a**) as a tool to follow hemoglobin-mediated lipid oxidation in washed cod mince. *Journal of Agricultural and Food Chemistry*, 52(24), 7214-7221.

Yan, X. J., Li, X. C., Zhou, C. X., & Fan, X. (1996). Preservation of fish oil rancidity by phlorotannins from *Sargassum kjellmanianum*. *Journal of Applied Phycology*, 8(3), 201-203.

Yuan, Y. V., Westcott, N. D., Hu, C., & Kitts, D. D. (2009). Mycosporine-like amino acid composition of the edible red alga, *Palmaria palmata* (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. *Food Chemistry*, 112(2), 321-328.

Figures Captions

Fig. 1. Effect of *F. vesiculosus* extract and fractions on the development of (a) rancid odour, (b) thiobarbituric acid reactive substances (TBARS), (c) sum of key volatile oxidation compounds (1-penten-3-ol, 1-octen-3-ol, hexanal, *cis*-4-heptenal, heptanal and 2,4-heptadienal) and (d) redness (*a** value) loss in washed cod model. *F. vesiculosus* extract and fractions were added at 300 mg/kg model and propyl gallate (PG) at 100 mg/kg model. 50% ethanol was used as the carrier solvent at 1% of the final model weight. The final pH and moisture of the models were 6.3 and 85.2%, respectively. Results are expressed as average ± standard deviation from duplicate samples.

Fig. 2. Effect of *F. vesiculosus* fractions on the development of (a) rancid odour, (b) thiobarbituric acid reactive substances (TBARS), (c) sum of key volatile oxidation compounds (1-penten-3-ol, 1-octen-3-ol, hexanal, *cis*-4-heptenal, heptanal and 2,4-heptadienal) and (d) redness (*a** value) loss in alkali-solubilised cod muscle protein isolates. *F. vesiculosus* fractions were added at 300 mg/kg model and propyl gallate (PG) at 100 mg/kg model. 50% ethanol was used as the carrier solvent at 1% of the final model weight. The final pH and moisture of the models were 6.3 and 81.2%, respectively. Results are expressed as average ± standard deviation from duplicate samples.

Table 1. Total phlorotannin content (TPC) and antioxidant activity of different *F. vesiculosus* extract and fractions

Extract/fractions	TPC (g PGE/100 g) [1)]	DPPH EC_{50} (μg/ml) [2)]	Fe^{2+} chelating ability (%)	Reducing power (mg ASE/g) [3)]
80% EtOH extract	35.4 ± 0.2^{c}	7.92 ± 0.11^{a}	34.7 ± 1.9^{bc}	324.8 ± 8.9^{b}
EtOAc fraction	88.3 ± 2.2^{b}	3.76 ± 0.22^{b}	34.2 ± 1.3^{c}	757.7 ± 38.2^{a}
LH-2 subfraction	96.6 ± 0.9^{a}	2.79 ± 0.05^{c}	28.5 ± 0.4^{d}	790.3 ± 61.3^{a}
LH-3 subfraction	97.0 ± 0.7^{a}	2.82 ± 0.03^{c}	30.5 ± 0.8^{d}	822.3 ± 29.2^{a}
LH-5 subfraction	91.3 ± 2.4^{b}	3.50 ± 0.13^{b}	38.0 ± 0.5^{b}	785.8 ± 77.1^{a}
LH-6 subfraction	87.5 ± 1.2^{b}	3.71 ± 0.07^{b}	41.9 ± 1.8^{a}	780.5 ± 82.1^{a}

Each value is expressed as means ± S.D. (n = 3).

Values in the same column followed by different superscript letters are significantly different ($P < 0.05$).

[1)] TPC: g phloroglucinol equivalents (PGE) /100g extract.

[2)] EC_{50}: concentration of extract (μg/ml) required to scavenge 50% of the DPPH• in the reaction mixture.

[3)] ASE, ascorbic acid equivalents.

Table 2. Partitioning of Sephadex subfractions between aqueous and oily phases

Subfractions	% in aqueous phase[1)]
LH-2	89.9 ± 2.6
LH-3	95.4 ± 2.1
LH-5	57.1 ± 2.3
LH-6	51.7 ± 1.5

Each value is expressed as means ± S.D. (n = 3).

[1)] phloroglucinol equivalents (PGE) in the aqueous phase divided by the total PGE before partitioning

Table 3. Volatile compounds identified by GC-MS and odour evaluation by GC-O in washed cod model system and cod muscle protein isolates.

		GC-MS[b]						GC-O[b]
		Washed cod muscle model system			Cod muscle protein isolate			
Compound	**RI[a]**	Control (Hb)	80% EtOH extract	EtOAc	Control (Hb)	LH-5	LH-2	**Odour description**
3-Methyl butanal	246		+					sweet, malty
1-Butanol	247		+	+	+	+	+	n.d.
1-Penten-3-ol	271	+	+	+	+	+	+	n.d.
2,3-Pentanedione	292	+	+	+	+	+	+	caramel
2-Pentenal	308	+	+	+		+		n.d.
2-Penten-1-ol	325	+	+	+	+	+		lemon, flowery
Unknown	351							sweet, buttery
Hexanal	394	+	+	+	+	+	+	grass
2-Hexenal	440	+				+		sweet
1-Hexanol	447	+	+	+		+		flowery
Unknown	469							earthy, mushroom
Unknown	484							buttery
cis -4-Heptenal	495	+	+	+	+	+		boiled potato-like
Heptanal	497	+	+	+	+	+		boiled potato-like
Unknown	524							flowery
Benzaldehyde	565	+	+	+	+	+		n.d.
Unknown	557							buttery
1-Octen-3-ol	582	+	+	+	+	+		mushroom
6-Methyl-5-hepten-2-one	590					+		flowery
Octanal	603	+	+		+			n.d.
2,4-Heptadienal	611	+	+	+	+	+		sweet, citrus
Unknown	634							flowery
Unknown	650							cardboard, sweet
Unknown	658							green
3,5-Octandien-3-one	670	+			+	+		fatty, sweet
Heptanoic acid	682		+	+				n.d.
3,5-Octadien-2-one	695	+		+		+		flowery
Nonanal	708	+	+	+		+		n.d.
Unknown	719							flowery
Unknown	747							cardboard, heavy
2,6-Nonadienal	756	+						cucumber, green
Octanoic acid	779		+	+				green, sweet
Decanal	816	+	+	+	+	+	+	sweet , fatty
Nonanoic acid	897		+	+				n.d.
2-Undecanone	900	+						flowery
Unknown	925							sweet

[a]Calculated ethyl ester index for DB-5ms capillary column; RI for unknown compounds based on GC-O analysis

[b]identified by: +, mass spectra; odor description, GC-O, RI and authentic standards.

Table 4. Fatty acid composition of washed cod muscle and cod muscle protein isolates [1)]

Fatty acid	Washed cod model	Cod protein isolates
C14:0	0.85 ± 0.07	1.05 ± 0.07
C16:0	18.50 ± 0.57	17.55 ± 0.78
C16:1*n*−9	0.15 ± 0.07	0.25 ± 0.07
C16:1*n*−7	0.85 ± 0.07	0.95 ± 0.07
C16:2*n*−4	0.35 ± 0.07	0.40 ± 0.00
C18:0	4.35 ± 0.07	3.45 ± 0.35
C18:1*n*−11	0.75 ± 0.07	1.35 ± 0.07
C18:1*n*−9	5.80 ± 0.14	6.25 ± 0.21
C18:1*n*−7	3.25 ± 0.21	2.45 ± 0.07
C18:1*n*−5	0.15 ± 0.07	0.20 ± 0.00
C18:2*n*−6	0.50 ± 0.00	0.70 ± 0.00
C20:0	0.05 ± 0.07	0.00 ± 0.00
C18:3*n*−3	0.20 ± 0.00	0.25 ± 0.07
C20:1*n*−11	0.20 ± 0.00	0.35 ± 0.07
C20:1*n*−9	1.25 ± 0.07	1.20 ± 0.14
C20:1*n*−7	0.10 ± 0.00	0.10 ± 0.00
C18:4*n*−3	0.35 ± 0.07	0.40 ± 0.00
C20:2*n*−6	0.10 ± 0.00	0.20 ± 0.00
C22:0	0.00 ± 0.00[6)]	0.00 ± 0.00
C20:3*n*−6	0.10 ± 0.00	0.10 ± 0.00
C22:1*n*−9	0.40 ± 0.00	0.55 ± 0.07
C20:4*n*−6	2.65 ± 0.21	3.45 ± 0.21
C20:4*n*−3	0.30 ± 0.00	0.40 ± 0.00
C22:2*n*−6	0.05 ± 0.07	0.20 ± 0.00
C20:5*n*−3	14.05 ± 1.20	12.75 ± 0.92
C24:1	0.45 ± 0.07	0.55 ± 0.07
C22: 5*n*−3	1.35 ± 0.07	1.75 ± 0.07
C22:6*n*−3	34.05 ± 2.76	34.70 ± 1.41
SFA[2)]	23.72 ± 0.57	22.11 ± 0.48
MUFA[3)]	13.38 ± 0.58	14.07 ± 0.63
PUFA[4)]	54.07 ± 4.26	55.26 ± 2.69
other	8.84 ± 5.42	8.55 ± 3.80

Results are expressed as average ± standard deviation from duplicate samples.

[1)] Fatty acid distribution expressed in peak area % of total fatty acid methyl esters (FAME).

[2)] SFA, saturated fatty acid; [3)] MUFA, monounsaturated fatty acid; [4)] PUFA, polyunsaturated fatty acid.

[6)] Values equal to 0.00 are values less than 0.10%.

Figure 1.

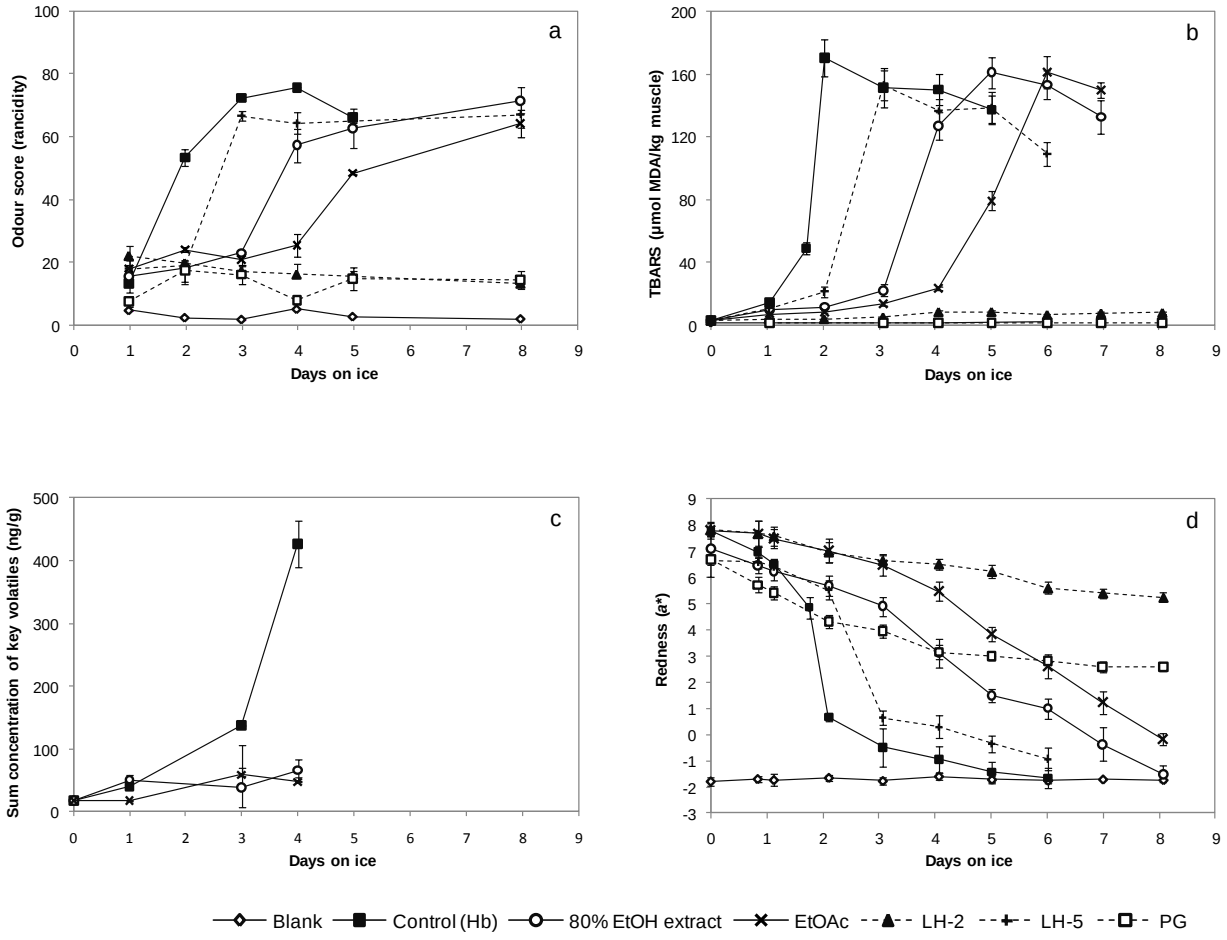
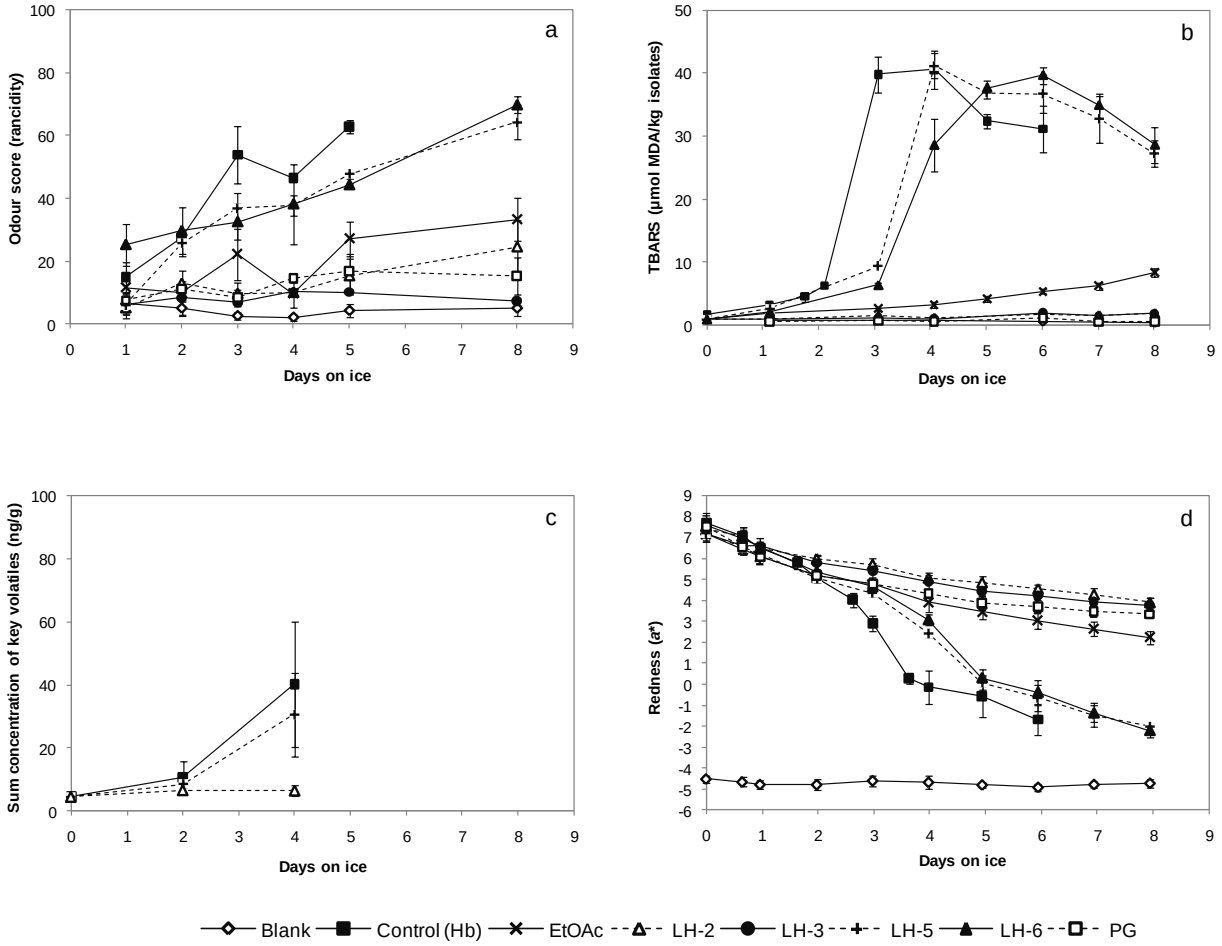

a
Odour score (rancidity)
Days on ice
b
TBARS (µmol MDA/kg muscle)
Days on ice
c
Sum concentration of key volatiles (ng/g)
Days on ice
d
Redness (a*)
Days on ice
Blank
Control (Hb)
80% EtOH extract
EtOAc
LH-2
LH-5
PG

Figure 2.


a
Odour score (rancidity)
Days on ice
b
TBARS (µmol MDA/kg isolates)
Days on ice
c
Sum concentration of key volatiles (ng/g)
Days on ice
d
Redness (a*)
Days on ice
Blank
Control (Hb)
EtOAc
LH-2
LH-3
LH-5
LH-6
PG