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Oxidation of marine lipids in liposomes and emulsions mediated by iron and methemoglobin

Thesis for the degree of Philosophiae Doctor

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Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
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Summary

Long chain omega-3 polyunsaturated fatty acids (LC omega-3 PUFA) are vital for physiological functions and have therapeutic and health benefits. The consumption of LC PUFA in the Western world has been below recommended intake levels the past decades, despite promotion of seafood and omega-3 supplements. Incorporation of the LC PUFA into processed food consumed on a daily basis might therefore bridge the gap between the recommended and actual consumption of LC omega-3 PUFA. Unfortunately, the development of omega-3 enriched food is hampered by a very high susceptibility of LC PUFA to oxidative deterioration. Furthermore, oxidised lipids are believed to create health risks. It has also been suggested that gastric juice may deteriorate the healthy LC PUFA after they are ingested. Important lipid oxidation promoters in food are low molecular weight (LMW) iron (Fe) and methemoglobin (metHb). To incorporate the LC omega-3 PUFA safely into food with respect to oxidation, it is necessary to understand both Fe- and metHb-mediated oxidation of PUFA and how the oxidation is influenced by conditions and dietary antioxidants.

The main objective of this thesis is therefore to study Fe- and metHb-mediated lipid oxidation in food-related lipid model systems – emulsions stabilised with phospholipids and liposomes made of phospholipids – containing LC omega-3 PUFA from fish. The focus was on clarifying the reaction mechanisms and the impact of different factors, including dietary antioxidants and gastric juice, on the prooxidant activity of Fe and metHb. Measurement of the consumption rate of the essential substrate for lipid oxidation – oxygen – by the LC PUFA was used for assessment of lipid oxidation.

The continuous measurement of the dissolved oxygen concentration has been shown to be a robust method for direct and instantaneous monitoring of peroxidation in both the liposomes and emulsions. The method was especially useful for measurement of the oxygen consumption kinetics in the lipid systems. The determination of oxygen uptake rates (OUR) enabled screening and evaluation of the impact of the different factors and antioxidants on the prooxidant activity of Fe and metHb.

Pre-formed lipid hydroperoxides (LOOH) were shown to be essential for the prooxidant activity of both Fe and metHb, and the prooxidant activity of metHb was not affected by the lack of light. The oxygen uptake kinetics revealed that iron behaved as a catalyst in lipid oxidation while the prooxidant activity of metHb weakened over time, presumably due to degradation of meHb molecule during lipid oxidation. MetHb was shown to be a stronger prooxidant than Fe, but the strong prooxidative activity was facilitated by a complete structure of the metHb molecule. The prooxidant mechanism of both Fe and metHb was not limited by the level of dissolved oxygen, as long as oxygen was present, or the level of pre-formed LOOH and double bonds in fatty acids, as long as they were present in higher concentrations than the prooxidant.

The extent of the prooxidative activity of Fe was shown to vary in dependence on:

- The total surface area: Smaller liposomal vesicles with lower lipid content were more prone to oxidation than larger emulsion droplets with a higher lipid content, presumably due to more frequent interactions of Fe with pre-formed LOOH in the interphase.
- The amount of phospholipid emulsifier: Higher levels of phospholipids resulted in the formation of smaller droplets. The highest OUR were measured for emulsifier concentrations ranging from 5 – 10% (w/w lipid base).
- pH of the aqueous phase: Fe-mediated oxidation was highest at pH interval 4.5 – 5.5.
- Dissolved compounds: Sodium chloride (NaCl) and 0.2% of xanthan gum dissolved in the aqueous phase inhibited Fe-mediated oxidation in a concentration dependent manner.

Electrostatic retention of Fe by phosphate groups within phospholipid heads has been suggested to facilitate the contact between pre-formed LOOH and Fe, and to create competitive reactions for iron precipitation at pH > 5 and iron complexation by chelating compounds.

The activity of dietary antioxidants has been shown to be affected by the type of prooxidant in the lipid system. Ascorbic acid, caffeic acid, propyl gallate, astaxanthin, ascorbyl palmitate, α-tocopherol, and δ-tocopherol inhibited metHb-mediated oxidation in concentration dependent manners. EDTA had a minor effect on metHb-mediated oxidation.

In Fe-mediated oxidation, caffeic acid, ascorbic acid and α-tocopherol were prooxidants. They directly interacted with Fe, reducing Fe^{3+} to the more catalytically active Fe^{2+}. The magnitude of the pro-oxidative behaviour was dependent on the Fe-to-antioxidant ratio, antioxidant concentration and pH. Ascorbic acid was depleted by interactions with Fe, and decreased the pro-oxidative activity of α-tocopherol. EDTA and citric acid inhibited Fe-mediated oxidation completely at twice the ratio to Fe and pH > 3.5. Propyl gallate efficiently inhibited Fe-mediated oxidation, while astaxanthin and β-carotene had only minor effects. In addition, chemical structure and physical location of the antioxidants determined their effects.

The work in this thesis shows that for correct interpretation of the effects of antioxidants it is important to assess what types of prooxidants are present in the system.

Both gastric juice and hydrochloric acid solution (HCl) did not prevent oxidation of marine lipids in emulsions and liposomes (pH 4.0). Furthermore, gastric juice did not inhibit metHb-mediated oxidation, but it was capable of reducing the prooxidant activity of dietary LMW iron, compared to HCl solution. Berry juice, green tea, red wine, and caffeic acid reduced the OUR in the acidic environments while coffee, ascorbic acid and orange juice increased the OUR. Therefore, beverages accompanying foods rich in marine lipids will affect the course of post-prandial lipid oxidation.

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28th February 2014, Trondheim

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List of papers

Paper I

Kristinova, V., Mozuraityte, R., Aaneby, J., Storrø, I., and Rustad, T. Iron-mediated peroxidation in marine emulsions and liposomes studied by dissolved oxygen consumption. *Eur. J. Lipid Sci. Technol.* 2014, 116 (2), 207–225.

Paper II

Kristinova, V., Mozuraityte, R., Storrø, I., and Rustad, T. Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants. *J. Agric. Food Chem.* 2009, 57 (21), 10377–10385.

Paper III

Kristinova, V., Aaneby, J., Mozuraityte, R., Storrø, I., and Rustad, T. Effect of dietary antioxidants on iron-mediated peroxidation in emulsions studied by dissolved oxygen consumption. *Eur. J. Lipid Sci. Technol.* 2014, 116 (–), 0000–0000 (Early View on-line)

Paper IV

Kristinova, V., Storrø, I., and Rustad, T. Influence of human gastric juice on oxidation of marine lipids – *in vitro* study. *Food Chemistry*, 2013, 141 (4), 3859–3871.

Additional contribution

PAPER V: Review

Medina, I., Undeland, I., Larsson, K., Storrø, I., Rustad, T., Jacobsen, C., Kristinova, V., and Gallardo, J. M. Activity of caffeic acid in different fish lipid matrices: A review. *Food Chemistry*, 2012, 131 (3), 730–740.

Authors' contributions

PAPER I – IV were initiated and edited by V. Kristinova. Experiments described in the papers were planned by V. Kristinova with input from R. Mozuraityte, I. Storrø and T. Rustad (PAPER I – IV), and J. Aaneby (PAPER I and III). All the experiments in PAPER II and IV and a part of the experiments in PAPER I and III were conducted by V. Kristinova. Some experiments were conducted by R. Mozuraityte (PAPER I) and J. Aaneby (PAPER I and III). PAPER I – IV were written by V. Kristinova. J. Aaneby contributed with pre-writing some sections in PAPER I and III.
PAPER V (Review) was initiated, edited, and written by I. Medina. V. Kristinova contributed with quality assurance and proof-reading.

Research presented in the section *Additional data* (page 53) was planned by V. Kristinova with input from R. Mozuraityte, I. Storrø and T. Rustad. All the experiments were conducted by V. Kristinova, except for laboratory work on methemoglobin-mediated oxidation in liposomes (section 5.1) which was mainly conducted by L. Škrabalová. L. Škrabalová also contributed with processing of the measured data, literature search and pre-writing some parts.

Abbreviations

$^1O_2$	singlet oxygen
$^3O_2$	triplet oxygen
ABTS	2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid
Ag	silver
ALA	α-linolenic acid
aox	antioxidant(s)
AsA	ascorbic acid
AsP	ascorbyl palmitate
BHT	butylated hydroxytoluen
C	carbon atom
CaA	caffeic acid
CAD	charged aerosol detector
CoA	*p*-coumaric acid
DHA	docosahexaenoic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
EPA	eicosapentaenoic acid
EDTA	ethylenediaminetetraacetic acid
Fe	iron
FeA	ferulic acid
FID	flame ionisation detector
FRAP	ferric reducing/antioxidant power
GC	gas chromatography
GI	gastrointestinal
H	hydrogen atom
$H^•$	hydrogen radical
H^+	hydrogen proton
Hb	hemoglobin
HCAD	hydroxycinnamic acid derivatives
HCl	hydrochloric acid
$HOO^•$	hydroxyperoxyl radical
HPLC	high pressure liquid chromatography
$L^•$	alkyl radical
LC PUFA	long chain polyunsaturated fatty acids
LMW	low molecular weight
LOOH	lipid hydroperoxide
$LO^•$	lipid alkoxy radical
$LOO^•$	lipid peroxyl radical
Mb	myoglobin
MDA	malondialdehyde
MES	2-(N-morpholino)ethanesulfonic acid
metHb	methemoglobin
metMb	metmyoglobin

NMR	nucleic magnetic resonance
O_2	oxygen
−OH	hydroxyl group
OUR	oxygen uptake rate(s)
PUFA	polyunsaturated fatty acid(s)
PG	propyl gallate
Pt	platinum
SET	single electron transfer
TBARS	thiobarbituric acid reactive substances
TBHQ	*tert*-butylhydroquinone
TLC	thin layer chromatography
Toc, Toc-OH	tocopherol
Toc-$O^{\bullet}$	tocopheroxyl radical
UV	ultraviolet
UV/VIS	ultraviolet/visible spectrophotometry

1 Introduction

Long chain omega-3 fatty acids (LC ω-3 PUFA) from marine organisms are vital for physiological functions in the human body and have numerous therapeutic and health benefits [1]. Docosahexaenoic acid (DHA) is mainly incorporated into the phospholipids of cell membranes and serves as a structural element, improving the membrane properties [2]. DHA plays a critical role in vision and cognitive functions and in the development of foetuses. Eicosapentaenoic acid (EPA) is involved in regulatory functions, such as gene expression and eicosanoid production [3]. It has been shown that EPA and DHA are preventive against the development of cardiovascular diseases [1].

In the Western world, the intake of EPA and DHA has been far below the recommended daily intake levels (RDI = 250 mg EPA+DHA/day, current daily intake estimate is < 100 mg EPA+DHA/day) [4], despite promotion of seafood and omega-3 supplements by various health organizations and dietary programs (for example matprat.no and godfisk.no in Norway). As shown for fish consumption [5], it is difficult to change eating habits of populations and preferences of individuals, and for some countries seafood may even be a luxurious commodity.

Incorporation of the LC omega-3 PUFA into processed food consumed on a daily basis might therefore bridge the gap between the recommended and actual consumption of LC omega-3 PUFA without major adjustments in the diet composition. Enrichment of various daily food with LC omega-3 PUFA (fish oil) is possible due to advances in food technology [6], but the fortification concept faces several challenges.

The main challenge is a very high susceptibility of LC omega-3 PUFA to oxidative deterioration which makes fish oils difficult to use as food ingredients. Lipid oxidation leads to development of off-flavours and unpleasant "fishy" aromas, and it shortens the shelf-life of fortified products which leads to issues with sensorial perception and marketing [7]. Therefore, the challenges to overcome are mainly related to the control of oxidation processes in complex food matrices [1, 8].

Another negative aspect of lipid oxidation is health risks associated with oxidised LC omega-3 PUFA. Oxidised derivatives of LC omega-3 PUFA are believed to be toxic and involved in the development of atherosclerosis, thrombosis and cancer [9, 10]. Lipid oxidation is unfortunately not restricted to food production and storage only. After the LC omega-3 PUFA are ingested, they are exposed to the environment of the stomach and intestinal tract. It has been proposed that LC omega-3 PUFA may further deteriorate in the gastrointestinal environment before they are metabolised [11]. Therefore, more studies on post-prandial oxidation of marine lipids are needed to elucidate this topic.

A large number of foods suitable for fortification with LC omega-3 PUFA is in the form of emulsions or is in an emulsified form at some time during the production (for instance, dairy and

bakery products, meat products, infant formulas, sauces or beverages). Systematic studies on lipid oxidation in variously complex emulsions have been undertaken during the past 30 years to better understand the oxidation processes in emulsions. Nevertheless, despite intensive research in this area, production of oxidatively stable food emulsions enriched with LC omega-3 PUFA is still problematic [12, 13]. A part of the problem is the complexity of food emulsions. Many factors may affect the rate and extent of lipid oxidation simultaneously and identifying the key factors in such multi-component and multi-phase systems is very difficult or even impossible [13].

LC omega-3 fatty acids are incorporated into emulsions as triacylglycerols (bulk oil) or phospholipids. Due to the amphiphilic character, phospholipids may serve not only as the source of the LC omgea-3 fatty acids, but also as a natural emulsifier. In addition, emulsion droplets stabilised with phospholipids may improve bioavailability of the emulsified lipids during digestion [14]. In this respect, marine phospholipids may have numerous functions in fortified food.

Low molecular weight (LMW) iron (also known as "free" or "ionic" iron) is a potent mediator of lipid oxidation even at trace levels. Unfortunately, free iron is a ubiquitous element in food and is considered the most common endogenous prooxidant in food emulsions [1, 15]. Another potent dietary prooxidant is hemoglobin which is found mainly in bodily organs and meat, including fish muscle [16]. Methemoglobin is generated as a result of blood hemoglobin degradation in *post mortem* tissue, and it therefore a likely type of hemoglobin to be present in muscle food.

One way to protect the LC omega-3 PUFA from oxidation is to add antioxidants. Even though antioxidant mechanisms of different dietary antioxidants are well described, the efficiency of the antioxidants in food is variable and needs to be better understood [13].

To incorporate the healthy LC omega-3 PUFA safely into food emulsions in respect to oxidation, it is necessary to understand both free iron- and methemoglobin-mediated oxidation of LC omega-3 PUFA in emulsified systems and how the prooxidant activity of these two dietary prooxidants is affected in emulsified systems. To develop effective strategies for stabilization of LC omega-3 PUFA against oxidation in emulsions with the use of antioxidants, the effects of dietary antioxidants on LMW iron- and metHb-mediated oxidation need to be characterized. Understanding individual factors which affect LMW iron- and metHb-mediated oxidation will help to better understand oxidation of LC omega-3 PUFA in more complex food emulsions.

2 Aim of the work

The main objective of this work was to study low molecular weight iron- and methemoglobin-mediated oxidation of marine lipids in food-related model systems – emulsions stabilised with phospholipids and liposome dispersions made from phospholipids – in order to better understand the behaviour of the two prooxidants in emulsified systems. Elucidation of the prooxidant mechanisms of the prooxidants, and how the prooxidant activity is affected by dietary antioxidants and various factors (incl. gastric juice) was in focus. The measurement of the dissolved oxygen consumption (oxygen uptake) by unsaturated fatty acids was used as a tool for studying iron- and methemoglobin-mediated oxidation of marine lipids.

The specific scientific objectives were to answer the following questions:

- Can the oxygen uptake method, used for studying iron-mediated lipid oxidation in liposome dispersions, be used for lipid oxidation studies in fish oil emulsions?
- What is the oxygen consumption kinetics in methemoglobin-mediated oxidation in the emulsified systems? What are the differences between oxygen uptake kinetics in iron- and methemoglobin-mediated oxidation?
- Is the prooxidant activity of iron in liposome dispersions comparable to prooxidant activity in emulsions?
- What is the impact of the system's properties on the prooxidant activity of iron? Factors, such as pH of the aqueous phase, unsaturation level of lipids, oxidative status of lipids, composition/size of emulsion droplets, and presence of other dietary compounds, such as salt and thickener, in the system were of interest.
- Do prooxidants affect the activity of antioxidants? Do dietary antioxidants interact with prooxidants? What is the impact on lipid oxidation in emulsified systems?
- Is lipid oxidation limited only to emulsions outside the human body? – Can human gastric juice aid lipid oxidation after the marine lipids are ingested? Does human gastric affects the prooxidant activity of iron and methemoglobin? Can post-prandial oxidation of marine lipids be influenced by antioxidants in beverages?

3 Background

3.1 Marine lipids and humans

3.1.1 Intake

Marine lipids is a collective term for a group of lipids originating from marine organisms and containing *n*-3 polyunsaturated fatty acids (PUFA) which contain a carbon chain counting C ≥ 20. These acids are also known as *long chain polyunsaturated fatty acids* (LC PUFA), *marine omega-3 fatty acids* or simply *omega-3*. The source of LC omega-3 PUFA in the diet is predominantly fatty fish and in some countries, such as Norway, tran (refined cod liver oil). Alternatively, supplements of high-quality fish oil, or food to which fish oil was added, so called *functional food* or *fortified food*, provide the marine omega-3 intake [17].

The human body is, to a certain degree, able to synthesize the most important LC omega-3 PUFA, eicosapentaenoic acid (EPA, 20:5 *n*-3) and docosahexaenoic acid (DHA, 22:6 *n*-3) (Figure 1), provided the body has sufficient intake of their precursor, the essential *α*-linolenic acid (ALA, 18:3 *n*-3). The dietary source of the latter is predominantly plants. It has been disputed whether it is possible for humans to produce adequate supplies of EPA and DHA from a sufficient supply of ALA, or whether these two acids must be part of a healthy diet. It has been estimated that ~5% of EPA and < 0.5% of DHA is converted from ALA, although these values may vary between for instance individuals who eat fish and non-fish eaters [18]. The ability to convert ALA is higher for women of reproductive age than for men, due to supply of DHA to the placenta and foetus during pregnancy, and to the milk during breast-feeding. [17, 19]

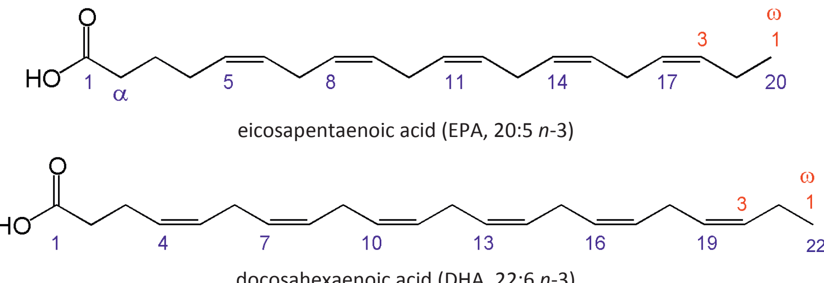

Figure 1 Structures of dietary important long chain omega- 3 polyunsaturated fatty acids – EPA and DHA – showing multiple double bonds interrupted by a methylene group (–CH_2–)

Recent daily intake recommendations for omega-3 fatty acids, EPA and/or DHA given by official organizations are rather inconsistent. In addition, the values are further specific for infants, small children and adults, elderly people, and pregnant/breastfeeding women. There is also a separate category for prevention of coronary heart and other diseases. Many organisations recommend regular fatty fish consumption over specific quantities of EPA and DHA (overview in Table 1) [4, 17, 20-22].

Table 1 Recommendations for intake of omega-3 fatty acids, EPA and DHA by various health organisations

Organisation	Recommendations for healthy people
Food and Agriculture Organization of the United Nations/The World Health Organisation (FAO/WHO) (2010)	Total omega-3 fatty acid intake = 0.5 – 2.0 E%* Adults = 250 mg EPA + DHA/day Pregnant/lactating adult women = 300 mg EPA + DHA/day, of which at least 200 mg should be DHA
Nordic Nutrition Recommendations (2004)	Total omega-3 fatty acid intake = 0.5 E% for children from 2 years of age and adults 1.0 E% for infants between 6 – 11 months and pregnant/lactating women
National Food Administration in Sweden (2007)	100 – 300 mg DHA/day for pregnant/lactating women
European Food Safety Authority (EFSA) (2010)	250 mg/day EPA + DHA/day for children from 2 years of age and adults
British Nutrition Foundation (2002)	1250 mg EPA + DHA/day
French Food Safety Agency (AFFSA) (2010) [22]	Young children (1 – 3 years): 70 mg EPA+DHA/day Children (3 – 9 years): 125 mg EPA/day + 125 DHA/day Adolescents (10 – 18 years) / Adults, incl. elderly / Pregnant women / Breastfeeding women: 250 mg EPA/day + 250 mg DHA/day
	Recommendations for primary prevention of coronary heart disease
FAO/WHO (2003)	1 – 2 servings of fatty fish per week (each serving providing equivalent of 200 – 500 mg EPA + DHA)
American Dietetic Association/Dieticians in Canada (2007)	2 servings of fatty fish per week
American Heart Association (2006)	2 servings of fatty fish per week
American Diabetes Association (2008)	2 or more servings of fatty fish per week
Australia and New Zealand National Health and Medical and Research Council (2006)	430 mg DHA/day for adults 610 mg EPA + DPA/ day for adults

Table 1 Recommendations for intake of omega-3 fatty acids, EPA and DHA by various health organisations (continuation)

EFSA (2010)	1 – 2 servings of fatty fish per week or 250 mg EPA + DHA/day
National Food Administration in Sweden (2007)	1 – 2 servings of fatty fish per week or 250 mg EPA + DHA/day
AFFSA (2010) [22]	500 mg EPA + DHA/day out of which at least 250 mg is DHA
European Society for Cardiology (2003)	Eating fatty fish
Health Council of the Netherlands (2010)	2 servings of fatty fish per week or 450 mg omega-3 fatty acids/day
Superior Health Council of Belgium (2004)	EPA + DHA = 0.3 E% for adults (approx. 667 mg/day)
International Society for the Study of Fatty acids and Lipids (2004)	500 mg EPA + DHA/day
United Kingdom Scientific Advisory Committee on Nutrition (2004)	2 servings of fatty fish per week or 450 mg EPA + DHA/day
Report from the National Council of Nutrition in Norway (2011)	Eating fatty fish or omega-3 supplements

* E% = per cent of total energy intake; sources: [4, 17, 20-22]

3.1.2 Physiological and health effects of marine lipids

LC omega-3 PUFA have a series of important physiological functions in the human body [1]. DHA is mainly incorporated into the phospholipids of cell/organelle membranes and serves as a structural element improving the membrane properties [2]. EPA is, in addition to being a structural element of membranes, involved in regulatory functions, such as gene expression and eicosanoid production [3].

Omega-3 fatty acids are required for normal conception, growth and development of embryos. During the third trimester of pregnancy, 50 – 60 mg/day of maternal DHA are transferred to the foetus via the placenta. DHA is highly concentrated in the brain and retinal membranes, especially in photoreceptors, and is therefore assumed to play a critical role in both vision and cognitive functions [3].

Therapeutic benefits in preventing and curing heart, coronary, mental and chronic diseases have been reported in a vast number of animal, epidemiological and clinical studies, and many of these studies have been extensively reviewed and critically evaluated [1, 3, 21, 23, 24]. Even though the research

on health benefits of omega-3 fatty acids has been intensive since their discovery in 70's, there is still a controversy whether healthy people benefit from dietary omega-3 fatty acids and whether the observed health benefits are manifested only in people with clinical symptoms. Nevertheless, it is more than clear that the intake of marine omega-3 fatty acids has a positive influence on human body and the beneficial effects have the potential to improve healthiness of individuals. For example, a reduction of the coronary heart disease risk is believed to be mediated through the ability of omega-3 fatty acids to lower heart rate and blood pressure, prevent arrhythmias and modify the plasma lipid profile by decreasing the levels of plasma triacylglycerols and low-density-lipoprotein (LDL) cholesterol [24].

However, marine lipids may, under certain conditions, have adverse health effects. It has been found that at high dosages (up to 7 g/day) bleeding time is increased and peroxidation of the fatty acids within chylomicrons (blood lipids) occurs. Taste perversion (so called "fishy taste") and some gastrointestinal disturbances have been associated with high intake of oily food in general [21].

Unfortunately, the modern (so called "Western") diet has evolved into a diet rich in plant and saturated fats with low proportion of seafood and marine oils. This has resulted in a high omega-6/omega-3 ratio in the diet, which is believed to be associated with development of many chronic diseases. The present Western diet has the omega-6/omega-3 ratio of 15/1 – 16.7/1 compared with the diet of our early ancestors, which was estimated to have the ratio of 1/1 [25].

The public awareness of health benefits of marine lipids increases due to various health campaigns (for instance, matprat.no and godfisk.no in Norway). Despite efforts of authorities to propagate seafood, fish especially, in the diet, the consumption of LC PUFA is still too low in modern societies ($\approx$ 100 mg/day) [4]. Therefore, production of omega-3 supplements as well as addition of marine lipids into daily food is greatly encouraged. This may help to increase the levels of EPA and DHA in the diet and thus contribute to better health of the population [6], and consequently reduce the overall costs for medical treatments and hospitalisation due to cardiovascular and other diseases.

3.1.3 Negative health effects of oxidized fatty acids

Owing to the polyunsaturated nature, the omega-3 fatty acids easily suffer oxidative deterioration [17] (a detailed description of lipid oxidation pathways is given in Section 3.2.2). Due to the oxidative deterioration, the quality of fish oils, seafood, and food fortified with LC omega-3 PUFA may become poor, which may bring negative aspects into the omega-3 diet.

The effect of dietary oxidised fat on human health has been far less explored than the effects of presumably intact marine lipids. Cellular, animal and human studies on this topic have been reviewed

by Turner *et al.* [10] The authors concluded that oxidised lipids have numerous harmful effects on health – for instance, increasing the risk of atherosclerosis, thrombosis and cancer. The damaging effects have been attributed to toxic and reactive lipid oxidation products, such as oxygenated α-, β-unsaturated aldehydes [9], malondialdehyde [26], or *trans*-4-hydroxy-2-hexenal [27].

Oxidised lipids could be responsible for the varying degrees of effectiveness and other discrepancies associated with supplementation of marine lipids in clinical studies; in other words, oxidised marine lipids may attenuate or even cancel the beneficial effects [10].

3.2 Lipid oxidation in food

3.2.1 Implications

Lipid oxidation is responsible for impaired food quality due to changes primarily in flavour and aroma, and secondarily in colour and texture [6]. Marine lipids are especially susceptible to oxidation, therefore production of food containing marine lipids, both naturally and fortified, is challenging for the food industry [28]. Low molecular weight compounds with low threshold values are produced during oxidation of marine lipids, which makes the food less acceptable or even unacceptable by consumers, and in case of pure marine oils unsuitable as food ingredient [29].

In addition, oxidation of long-chain polyunsaturated fatty acids destroys the essential fatty acids and results in a generation of cytotoxic and genotoxic compounds which are believed to create health risks [9, 10]. The free radicals generated during oxidation may also co-oxidize other compounds, such as saccharides, proteins [30], vitamins etc., affecting the overall nutritional quality of food [31]. Lipid oxidation may occur at any stage during food processing, handling and storage, therefore development of efficient and long-lasting strategies for minimizing lipid oxidation is desired.

In order to overcome these barriers it is necessary to understand the factors and mechanisms involved in oxidation of LC omega-3 PUFA in food. Understanding the impact of both dietary prooxidants and antioxidants is of great importance. Prooxidants are ubiquitous in food, in trace or significant amounts, while antioxidants are usually added during production to hinder oxidation, but also may be present endogenously.

3.2.2 Theoretical aspects

Lipid oxidation is a term for a highly complex set of free radical reactions, where lipid hydroperoxides (LOOH) play a pivotal role as a primary oxidation product. A scheme of *fundamental* radical reactions involved in lipid oxidation is shown in Figure 2.

Three stages are typically associated with oxidation – initiation, propagation and termination [32].

3.2.2.1 Initiation

Initiation of lipid oxidation, *i.e.* producing the *ab initio* alkyl radical ($L^\bullet$) (reaction 1 in Figure 2), is not entirely understood. Thermodynamically, molecular oxygen (O_2) cannot react directly with double bonds because the spin states are different – oxygen is in triplet state ($^3O_2$), whereas the double bond is in a singlet state. Initiators are therefore required to remove an electron from either

the lipid or oxygen, forming a radical, or to change the electron spin of the oxygen, forming highly reactive singlet oxygen ($^1O_2$). [32]

Initiation (*formation of ab initio lipid free radical*)

$$L_1H \xrightarrow{k_i} L_1^{\bullet} \qquad (1)$$

Propagation

Free radical chain reaction established

$$L_1^{\bullet} + O_2 \underset{k_\beta}{\overset{k_o}{\rightleftharpoons}} L_1OO^{\bullet} \qquad (2)$$

$$L_1OO^{\bullet} + L_2H \xrightarrow{k_{p1}} L_1OOH + L_2^{\bullet} \qquad (3)$$

$$L_2OO^{\bullet} + L_3H \xrightarrow{k_{p1}} L_2OOH + L_3^{\bullet} \text{ etc. } \dashrightarrow L_nOOH \qquad (4)$$

Free radical chain branching (initiation of new chains)

$$L_nOOH \xrightarrow{k_{d1}} L_nO^{\bullet} + OH^- \text{ (reducing metals)} \qquad (5)$$

$$L_nOOH \xrightarrow{k_{d2}} L_nOO^{\bullet} + H^+ \text{ (oxidizing metals)} \qquad (6)$$

$$L_nOOH \xrightarrow{k_{d3}} L_nO^{\bullet} + {}^{\bullet}OH \text{ (heat and uv)} \qquad (7)$$

$$\left.\begin{matrix} L_nO^{\bullet} \\ L_nOO^{\bullet} \\ HO^{\bullet} \end{matrix}\right\} + L_4H \xrightarrow[k_{p1},\ k_{p3}]{k_{p2}} \left.\begin{matrix} L_nOH \\ L_nOOH \\ HOH \end{matrix}\right\} + L_4^{\bullet} \qquad (8a)\ (8b)\ (8c)$$

$$L_1OO^{\bullet} + L_nOOH \xrightarrow{k_{p4}} L_1OOH + L_nOO^{\bullet} \qquad (9)$$

$$L_1O^{\bullet} + L_nOOH \xrightarrow{k_{p5}} L_1OH + L_nOO^{\bullet} \qquad (10)$$

Termination (*formation of non-radical products*)

Radical recombinations

$$\left.\begin{matrix} L_n^{\bullet} \\ L_nO^{\bullet} \\ L_nOO^{\bullet} \end{matrix}\right\} + \left.\begin{matrix} L_n^{\bullet} \\ L_nO^{\bullet} \\ L_nOO^{\bullet} \end{matrix}\right\} \xrightarrow[k_{t2},\ k_{t3}]{k_{t1}} \text{polymers, non-radical monomer products (ketones, ethers, alkanes, aldehydes, etc.)} \qquad (11a)\ (11b)\ (11c)$$

Radical scissions

$$\left.\begin{matrix} LOO^{\bullet} \\ LO^{\bullet} \end{matrix}\right\} \xrightarrow[k_{ts2}]{k_{ts1}} \text{non-radical products (aldehydes, ketones, alcohols, alkanes, etc.)} \qquad (12a)\ (12b)$$

i - initiation; o - oxygenation; β - O_2 scission; p - propagation; d - dissociation; t - termination; ts - termination/scission

Figure 2 Fundamental free radical reactions involved in lipid oxidation (figure from [32]).

Several initiators have been identified for these processes:

- ultraviolet light (*hv*)
- photosensitizers
- metals
- heat
- ozone
- free radicals
- lipoxygenases (production of LOOH)

The ease of formation of the *ab initio* alkyl radical ($L^{\bullet}$) increases with increasing unsaturation. In PUFA, the double bonds are interrupted by a methylene group ($–CH_2–$) (Figure 1). Since the C–H covalent bonds of the methylene carbon are weakened by two adjacent double bonds, their bond dissociation energy is lower, and hydrogen abstraction becomes easier. [33] This is the reason for the high susceptibility of PUFA to oxidation.

With respect to initiation, alkyl radical formation due to UV irradiation occurs at wavelengths < 200 nm, where photon energy is sufficient to cleave covalent C–H and C–C bonds into free radicals [32].

Pigments in foods, such as chlorophylls, porphyrins (found in heme-proteins), and riboflavin, aromatic amino acids, and molecules with an extended conjugated double bond system (*e.g.* xanthene [34]) have photosensitizing properties, *i.e.* ability to convert energy of light into chemical energy [17, 32]. In food lipids, the *singlet oxygen ($^1O_2$) reaction pathway* mainly occurs. The sensitizer in singlet ground state (^{1}Sen) becomes excited (^{1}Sen*) by absorbing light energy (*hv*), and by intersystem crossing (ISC), the excited singlet sensitizer is converted into an excited triplet state sensitizer (^{3}Sen*). The energy of the excited triplet state sensitizer is transferred to $^3O_2$ to form $^1O_2$ while the sensitizer returns to its ground singlet state (^{1}Sen) (eq. 13). Electrophilic $^1O_2$ can then directly react with high-electron-density double bonds forming LOOH [31].

$$^1\text{Sen} \xrightarrow{hv} {}^1\text{Sen}^* \xrightarrow{\text{ISC}} {}^3\text{Sen}^* \xrightarrow{^3O_2} {}^1\text{Sen} + {}^1O_2 \qquad \text{(eq. 13)}$$

Under favourable conditions, higher valence metals ($M^{(n+1)+}$) can directly remove an electron from a double bond forming the *ab initio* alkyl radical (eq. 14). It has been found that the electron transfer is exothermic, *i.e.* energy is released upon the reaction, and extremely rapid in non-polar media, including pure oils. [32]

$$LH + M^{(n+1)+} \rightarrow L^{\bullet} + H^{+} + M^{n+} \qquad \text{(eq. 14)}$$

Lower valence metals (M^{n+}) can initiate oxidation through formation of activated complexes with O_2 or through autoxidation. Autoxidation of reduced metals generates oxygen radicals that then react with lipids. [32]

$$M^{n+} + O_2 \rightarrow M^{(n+1)+} + O_2^{-\bullet} \xrightarrow{H^+} HOO^{\bullet} \xrightarrow{LH} L^{\bullet} + H_2O_2 \qquad \text{(eq. 15)}$$

High temperatures (*e.g.* frying temperatures) have sufficient energy to cleave covalent C–H and C–C bonds and form the *ab initio* alkyl radicals. Atmospheric ozone (O_3) adds directly to double bonds to form ozonides, which then undergo a number of subsequent reactions, yielding alkyl ($L^{\bullet}$), alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals.

Radicals generated from various side reactions can lead to generation of the *ab initio* alkyl radical. Hydroxyl radicals ($HO^{\bullet}$) are the strongest oxidizing radicals. They attack fatty acids indiscriminately at all sides along the acyl chains. The half-life of $HO^{\bullet}$ is however extremely short (10^{-9} s) and they most likely react with adjacent solvent molecules, producing other and longer living radicals that can attack lipids. Lifetimes and hydrogen abstraction rates of various radicals are listed in Table 2. $O_2^{-\bullet}$ radical, which is often present in aqueous environment, does not react with lipids but it can be a source of the highly reactive $HO^{\bullet}$ and moderately reactive $HOO^{\bullet}$.

Lipoxygenases are often overlooked as initiators of lipid oxidation, even though they are present in all plant and animal tissues. The enzyme catalyses aerobic oxidation of fatty acids to form LOOH without a release of lipid radicals. LOOH produced by lipoxygenases can accumulate to relatively high levels, which then can trigger oxidation when LOOH are decomposed into radicals by heat, light or metals.

It is assumed that several initiators are always operative simultaneously, and only trace (< micromolar) quantities are required for generation of the *ab initio* radicals. Elimination, or at least inhibition, of production of these radicals is suggested to be the key strategy for attaining a long term stability of any material. However, for the reasons mentioned above, control over the initiation is impossible from a practical standpoint. Once the *ab initio* radicals are formed, they start the propagation. Consequently, lipids are always oxidized to some degree. The formation of the *ab initio* radicals is often "non-visible" and very difficult to detect. Therefore, initiation of lipid oxidation is sometimes wrongly perceived as "spontaneous". [32]

Table 2 Lifetimes and hydrogen abstraction rates of various radicals that can initiate lipid oxidation (figure from [32]).

Radical	Half-life with Typical Substrate, 10^{-3} M, 37°C		Ave. rx Rate, k (L mol^{-1}sec^{-1})	
$HO^{\bullet}$	10^{-9} sec		10^9–10^{10}	
$RO^{\bullet}$	10^{-6} sec		10^6–10^8	
$ROO^{\bullet}$	10 sec		10^1–10^3	
$L^{\bullet}$	10^{-8} sec		10^4–10^8	
$AnOO^{\bullet}$	10^{-5} sec			
$O_2^{-\bullet}$			~ 1	
$HOO^{\bullet}$			10^0–10^3	
	18:1	**18:2**	**18:3**	**20:4**
$HO^{\bullet}$	$\sim 10^9$	9.0×10^9	7.3×10^9	$\sim 10^{10}$
Monomer		8.0×10^9	8.0×10^9	
Micellar		1.3×10^9	2.5×10^9	
Non-allylic H	4×10^2	3.4×10^3	7.0×10^3	1.0×10^4
$RO^{\bullet}$	3.3×10^6	8.8×10^6	1.3×10^7	2.0×10^7
t-BuO$^{\bullet}$	3.8×10^6	9.1×10^6	1.3×10^7	2.1×10^7
	(*trans*) 3.3×10^6	(*trans*) 8.8×10^6		
aqueous	6.8×10^7	1.3×10^8	1.6×10^8	1.8×10^8
$ROO^{\bullet}$	1.1	6×10^1	1.2×10^2	1.8×10^2
$O_2^{-\bullet}$	no rx	no rx	<1	<1
	(MLOOH) 7.4×10^3			
$HOO^{\bullet}$	no rx.	1.1×10^3	1.7×10^3	3.1×10^3
		$<3 \times 10^2$		
O_3 –CCl_4	6.4×10^5	6.9×10^5		
–aq SDS	9.5×10^5	1.1×10^6		
$SO_3^{-\bullet}$		1.8×10^6	2.8×10^6	3.9×10^6
$GS^{\bullet}$	$<2 \times 10^6$	8×10^6	1.9×10^7	3.1×10^7
$^1O_2$	0.74×10^5	1.3×10^5	1.9×10^5	2.4×10^5
$O^{-\bullet}$	7.5×10^2	9.7×10^3	1.2×10^4	1.9×10^4
$NO_2^{\bullet}$	1.2×10^6	6.2×10^6	6.6×10^6	

[a]Aqueous solution.
[b]H abstraction from unsaturated alkenes.

3.2.2.2 Propagation

Propagation of oxidation happens via multiple pathways. The classical pathway is referred to as *autoxidation* (eq. 2, 3 and 4 in Figure 2). Hydrogen abstraction by $LOO^{\bullet}$ is however relatively slow and selective giving plenty of room for alternative pathways, which compete with autoxidation. This is the reason for complicated lipid oxidation kinetics and an extreme variety of oxidation products.

The competing reactions to autoxidation are:

- atom or group transfer (hydrogen abstraction),
- rearrangement/cyclization,
- additions to double bonds leading to crosslinks,
- disproportionation,
- β-scission,
- recombination,
- electron transfer ($LOO^{\bullet} + e^{-} \rightarrow LOO^{-}$), *e.g.* metal-catalysed propagation.

These mechanisms contribute to chain propagation, branching, isomerization and termination reactions. Which mechanism is prevailing depends strongly on reaction conditions, particularly on temperature. [32]

Lipid mono hydroperoxides (LOOH) are the key products of lipid oxidation and the only products of autoxidation, and are therefore perceived as the *primary oxidation products*. Some of the competing reactions may however generate various derivatives of LOOH (*e.g.* polyperoxides, epidioxides, hydroperoxy epidioxides, peroxidized polymers) and other compounds (*e.g.* epoxides and alcohols), which are often neglected as primary oxidation products.

The competing reactions mainly operate with alkoxyl ($LO^\bullet$) and peroxyl ($LOO^\bullet$) radicals generated by LOOH decomposition. Most of these radicals abstract hydrogens and propagate oxidation. The reactions lead to formation of various aldehydes, ketones, acids, alcohols, short chain hydrocarbons, etc., commonly grouped as *secondary oxidation products*.

The β-scission of alkoxyl radicals cleaves the aliphatic chain of the fatty acid producing typically an aldehyde and an alkyl radical. It is the main pathway responsible for decomposing fatty acids into low molecular weight compounds that are volatile enough to be perceived as oxidative rancidity. In case of PUFA, decomposition products can be unsaturated and have intact unsaturated structures, meaning that the oxidation products can be further oxidized or cleaved. [32, 33]

Lipid hydroperoxides accumulate in lipids over time. The following factors cause decomposition of LOOH into alkoxyl ($LO^\bullet$) and peroxyl ($LOO^\bullet$) radicals, which consequently branch the propagation and accelerate the oxidation:

- heat,
- metals,
- heme-compounds,
- UV light,
- peroxyl radicals,
- nucleophiles.

One or more of these decomposing factors are nearly always present. Metal- and heme-catalyzed oxidation is more closely described in Section 3.2.4.

The reaction pathways for lipid oxidation change with the type of reaction system, system conditions and with numerous other factors (*e.g.* solvent polarity, oxygen concentration and temperature).

It has been established that alkoxyl ($LO^\bullet$) radicals react several orders of magnitude faster than peroxyl ($LOO^\bullet$) radicals, and that these radicals abstract hydrogen atoms much faster from LOOH

than from allylic positions. It has also been established that all the above mentioned propagation pathways happen simultaneously and only small modifications in reaction conditions are sufficient to shift the balance between them.

Shifting among propagation pathways critically affects the kinetics of oxidation and product distributions, which has consequences for monitoring progress of oxidation. Without information about dominant and active propagation pathways, the most effective antioxidant strategies may not be applied. It has been suggested to control all active propagation pathways with antioxidant strategies to achieve long-term stability of lipids. [32]

3.2.2.3 Termination

Termination refers to quenching of individual radicals, forming non-radical products (eq. 11 and eq. 12 in Figure 2), but not to stopping the overall chain reaction. Free radicals are quenched by four major mechanisms:

- radical recombinations
- radical scissions
- radical transfer (co-oxidation of non-lipid molecules)
- eliminations

The mechanisms dominating in a given system are influenced by the nature and concentration of the radicals, temperature, oxygen pressure, and solvent.

The number of variations for radical recombination is nearly limitless, which leads to a broad range of secondary oxidation products. Scissions of alkoxyl radicals are the major source of aldehydes, which are responsible for flavour and odour.

One of the best known scission products of lipid oxidation is *malondialdehyde* (MDA) – a scission product from five-membered cyclic hydroperoxides, which can only be formed in linolenic acid (18:3 *n*-3) and PUFA. Formation of MDA first requires appropriate conditions to generate cyclic peroxide precursors, and then conditions for cleavage of the endoperoxides. [32]

Alkoxyl and peroxyl radicals can abstract hydrogen from non-lipid molecules, such as amino acids, nucleic acids, antioxidants, carotenoids and other pigments, and even carbohydrates. Radicals transferred to these non-lipid molecules may follow processes similar to lipids and in this way, lipids transfer oxidation to other molecules [30].

Radicals formed in non-lipid molecules may combine with lipid radicals to generate co-oxidation adducts. These adducts may not be extracted and included into lipid analysis. This has been often neglected in respect to lipid analysis of foods and biological systems. Lipid radical adducts to amino acids are important flavour precursors [32].

3.2.3 Oxidation of phospholipids

Phospholipids are a class of lipids containing a phosphoric acid residue. A phospholipid molecule consists of a polar/hydrophilic part (head) composed of glycerol, phosphate and a specific substituent, and a non-polar/lipophilic part (tail) composed of fatty acids (the basic structure is shown in Table 3). Due to their amphiphilic character, phospholipids are major components of biological membranes, and act as emulsifiers in food by adsorbing at the interface of aqueous and oil phases, thus lowering the interfacial tension [35].

Table 3 Basic classification of phospholipids

Basic structure	Substituent		Name of phospholipid	
R_1–O–CH_2 R_2–O–CH CH_2–O–P(=O)(O^-)–O–X R_1, R_2 … fatty acids Glycerol Phosphate group X … substituent	**Hydrogen atom**	–H	*Phosphatidic acid*	PA
	Choline	$-CH_2-CH_2-N^+(CH_3)_3$	*Phosphatidylcholine*	PC
	Ethanolamine	$-CH_2-CH_2-NH_3^+$	*Phosphatidylethanolamine*	PE
	Serine	$-CH_2-CH(COO^-)(NH_3^+)$	*Phosphatidylserine*	PS
	Glycerol	$-H_2C-HC(OH)-H_2C-OH$	*Phosphatidylglycerol*	PG
	Inositol	cyclohexane ring (C1–C6) with HO, OH, OH, HO, OH	*Phosphatidylinositol*	PI

It has recently been shown that in addition to the classical autoxidation and oxidation mediated by prooxidants, phospholipids are subjected to further degradation as a result of peroxidation of fatty acids. These subsequent reactions are characterized as *non-enzymatic browning* of phospholipids.

In non-enzymatic browning reactions, the primary oxidation products or their degradation products (aldehydes) react with the amine group of phosphatidylethanolamine, forming highly coloured pyrroles. [36, 37]. These compounds have not been detected in oxidation of triacylglycerols [38] and might be used in the future as markers for evaluation of oxidative status in phospholipids.

3.2.4 Prooxidants

3.2.4.1 Metals

Redox-active metals undergoing one-electron transfers (Fe, Cu, Co, Mn, Mg, Cr and V) are considered to be of the greatest importance for lipid oxidation in oils and foods because they are ubiquitous (ever present) in food and active in many forms, even at trace (nanomolar) concentrations. Redox-active metals break the O−O bond in LOOH by electron transfer to generate radicals and ions. Oxidising metals ($M^{(n+1)+}$) decompose LOOH at rates several orders of magnitude slower than reducing metals (M^{n+}) (eq. 16 and eq. 17).

Reducing metals: $M^{n+} + LOOH \rightarrow \text{(fast)}\ M^{(n+1)+} + LO^{\bullet} + OH^{-}$ $K \sim 10^{9}\ M^{-1}s^{-1}$ (eq. 16)

Oxidizing metals: $M^{(n+1)+} + LOOH \rightarrow \text{(slow)}\ M^{n+} + LOO^{\bullet} + H^{+}$ $K \sim 10^{4}\ M^{-1}s^{-1}$ (eq. 17)

Red-ox cycling: $2\ LOOH \xrightarrow{Fe} LO^{\bullet} + LOO^{\bullet} + H_2O$ (eq. 18)

The abundance of low molecular weight (LMW) ferric iron ($Fe(H_2O)_6{}^{3+}$) and ferrous iron ($Fe(H_2O)_6{}^{2+}$), also known as *free iron* or *ionic iron*, in food makes its prooxidant activity highly important. The prooxidant effect of iron is immensely amplified when red-ox cycling occurs, *i.e.* one iron atom "oscillates" between its Fe^{2+} and Fe^{3+} valence state. In this respect iron behaves as a catalyst of LOOH decomposition (eq. 18); this pathway is considered predominant when LOOH are in excess of LMW iron [32]. This requirement is almost always fulfilled in foods because food processing generates oxidative stress. Mozuraityte *et al.* [39] confirmed red-ox cycling of LMW iron in liposomes made of cod roe phospholipids and concluded that in this system one red-ox cycle is accompanied by consumption of five O_2 molecules by the generated radicals. The scheme for iron-mediated hydroperoxide decomposition is given in Figure 3.

Degradation of enzymes, pigments and metalloproteins during processing of raw materials as well as ingredients, tap water (containing ~ 0.2 mg Fe/L) and well/mineral water (containing > 680 mg Fe/L) [40], steel processing equipment and packing materials may release ionic iron into food matrices.

Iron is found endogenously in food but may also be added exogenously to increase the nutritional value of the food. Recommended daily intakes (RDI) of dietary iron for healthy infants are 1 mg/kg/day and for children, men and women the RDI varies between 10 – 15 mg/day [41]. Therefore various foods, such as bakery and dairy products, infant formulas, beverages, and even ingredients (salt, sugar, flour) may be enriched with iron. The iron content in certain products may reach values up to 500 mg/kg – for more details, see a review paper by Martínez-Navarrete *et al.* [41]

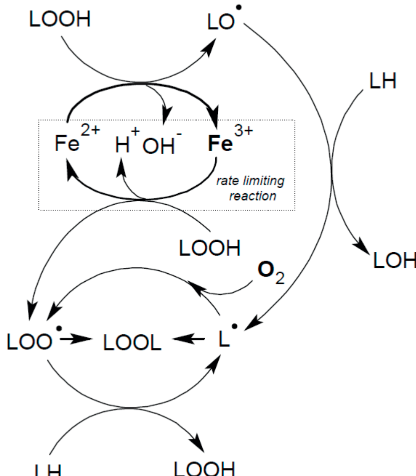

Figure 3 Iron-mediated lipid hydroperoxide decomposition (red-ox cycling of iron); LOOH = lipid hydroperoxide, $LOO^{\bullet}$ = lipid peroxyl radical, $LO^{\bullet}$ = lipid alkoxyl radical, $L^{\bullet}$ = lipid alkyl radical, LH = fatty acid, LOOL and LOH = lipid oxidation products

3.2.4.2 Heme-proteins

Heme-compounds (hemoglobin, myoglobin, catalase, peroxidase), *i.e.* proteins containing a porphyrin structure with embedded iron atom (Figure 4), are known to catalyse lipid oxidation at much higher rates than LMW iron. Hemoglobin and myoglobin are typically present in muscles (meat incl. fish) and tissues rich in blood, such as organs [16].

In living tissues, hemoglobin exists basically in the reduced form (Fe^{2+}) either saturated with an oxygen molecule (oxyhemoglobin), or desaturated of oxygen molecules (deoxyhemoglobin). After death (*post mortem*), the concentration of oxy- and deoxy-hemoglobin progressively decreases as it is converted (oxidised) to methemoglobin which cannot bind oxygen. Therefore methemoglobin is the likely hemoglobin species to be involved in peroxidation of *post mortem* tissues.

Studies on the reaction mechanism of heme-mediated oxidation indicate that an intact porphyrin-Fe structure inside a pocket formed by surrounding proteins is an absolute requirement for heme-catalysed lipid oxidation, and that hypervalent iron complexes – mainly ferryl iron complexes ($Fe^{4+}=O$ and $Fe^{4+}(OH)$) – are responsible for the rapid catalysis. The basic reaction mechanism involves binding of preformed LOOH to Fe^{3+}-heme which generates the ferryl iron complex in a very fast reaction ($K \sim 10^{9}\ M^{-1}s^{-1}$). The LOOH is then decomposed either heterolytically or homolytically inside the heme-pocket, producing lipid alcohols (R–OH) or $LO^{\bullet}$, respectively. The

resulting ferryl iron complex is an extremely strong oxidant and rapidly abstracts H from either a new LOOH or the fatty acid directly, which generates lipid radicals for chain reactions. The hypervalent iron complexes can be maintained by electron transfers, thus keep their oxidizing power. Eventually, they are reduced back to Fe^{3+}-hemes. The overall mechanisms of heme-catalyzed lipid oxidation is shown in Figure 5 [32].

The composition and arrangements of amino acids in the heme-pocket, as well as heme pocket size and orientation, affect lipid binding and proton abstraction, while the protein structure and ligands influence electron transfer processes and stabilisation of the ferryl iron complex. The reaction environment influences whether the LOOH cleavage is homolytic or heterolytic. Therefore variable catalytic activity between different heme-compounds and the same heme-compounds from different animals has been observed.

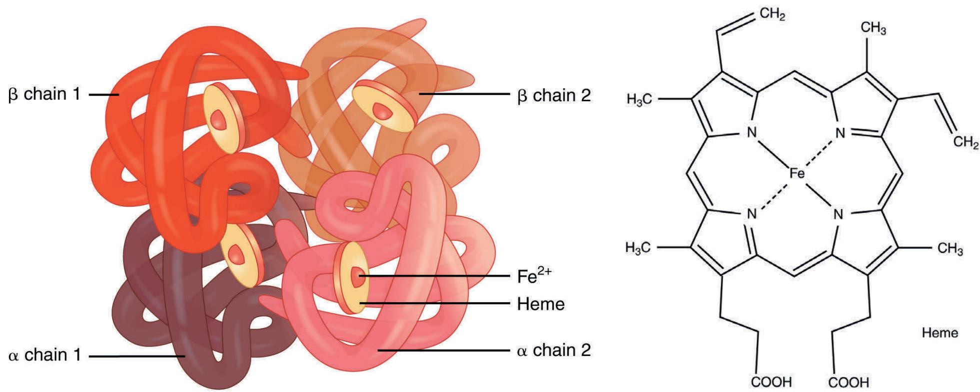

Figure 4 Left: Hemoglobin molecule consisting of four protein subunits (α-chain 1 and 2, β-chain 1 and 2), each unit containing a heme group; **Right:** Heme group = porphyrin structure with embedded iron atom (also known as *hemin* if the group is loose and the iron is Fe^{3+}); figure retrieved from: http://commons.wikimedia.org/wiki/File:1904_Hemoglobin.jpg

In Section 3.2.2.1, it is mentioned that porphyrin structures are capable of producing singlet oxygen via photosensitisation. This mechanism may contribute to oxidation in processed food where the whole heme-compound may be disintegrated and at the same time the heme-group is exposed to light, *i.e.* located on the surface of the product [32].

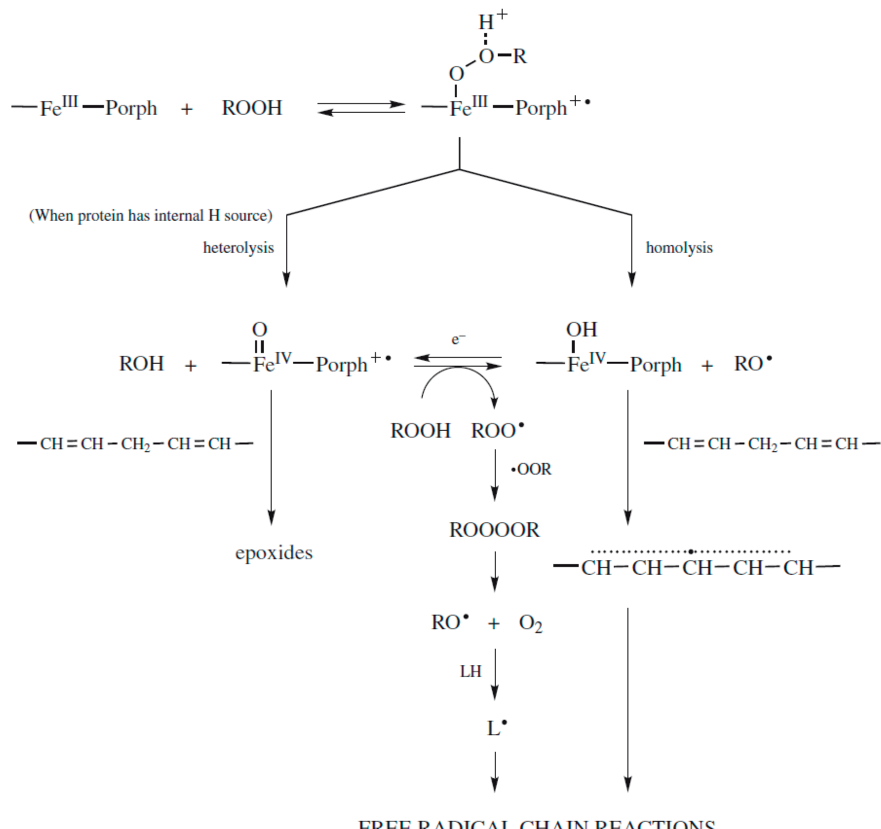

Figure 5 Heme-catalysed lipid oxidation via ferryl-iron complexes (figure from [32])

3.2.5 Lipid models for studying lipid oxidation

Studying lipid oxidation in food is often complicated due to the complex nature of most food matrices. Many components can have an impact on lipid oxidation simultaneously and identification of the key factors affecting lipid oxidation can be very difficult, or even impossible. Consequently, the interpretation of measured data may be erroneous or misleading. Using simpler systems which allow looking at individual factors separately may help to better understand these complex systems.

Most food fortified with omega-3 lipids would be in the form of emulsions, because fish oil can be evenly distributed throughout the volume as globules (emulsion droplets). Alternatively, marine omega-3 can be added to the food in the form of emulsions or liposomes and dispersed in the matrix. Dairy products, such as milk, and yoghurt, mayonnaise, spreadable fats, dressings, and beverages, such as juices, bakery products (bread), sausages and sauces are examples of daily food fortified with omega-3 available on the world's market [6].

In this thesis liposomes and emulsions were used as food-related emulsified systems for studying lipid oxidation. These systems allow manipulation of lipid composition, pH and content of various compounds in a controlled way [42].

3.2.5.1 Liposomes

A *liposome*, in its simplest form, is a microscopic spherical structure composed of a phospholipid bilayer enclosing an aqueous compartment (Figure 6). The liposome vesicle can range in size from 10 nm to 10 μm in diameter and be composed of more than one concentric phospholipid bilayer, depending on the method of preparation [42]. Due to the bilayer vesicular structure liposomes resemble cell membranes. The only lipids in lean fish muscle are the phospholipids of the cell membranes. Therefore, the liposome model may to a certain degree mimic oxidation of lean fish meat. Liposomes have also been related to cell membranes in a number of *in vitro* biological studies [43, 44], and in systematic studies on the activity of various pro- and antioxidants [45, 46].

The main applications of liposomes have been in pharmacology, as a drug delivery system, and in cosmetics [47]. In food, liposomes have been used scarcely – as carriers or for encapsulation of important nutrients, such as vitamins, peptides, enzymes etc. Liposomes might be the future delivery system of omega-3 phospholipids into food [48], or oral supplements of LC omega-3 PUFA [49].

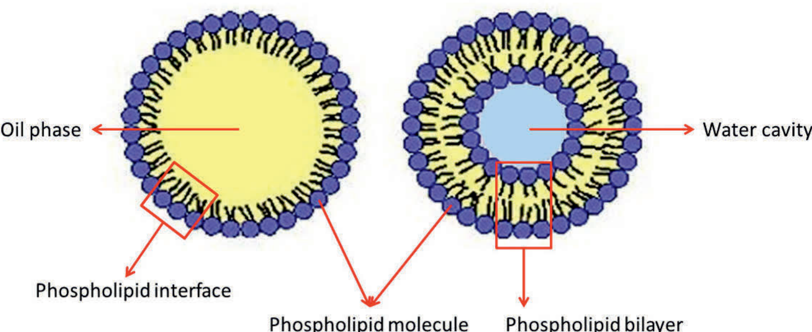

Figure 6 Cross-section of an emulsion droplet stabilised with phospholipids (**left**) and a liposome vesicle (**right**)

3.2.5.2 Emulsions

Most lipids in food exist as dispersions and emulsions. Emulsions are either water dispersed in oil (water-in-oil emulsion (w/o), *e.g.* margarines) or oil dispersed in water (oil-in-water emulsion (o/w), *e.g.* milk). The latter type is more common in food.

In kinetically stable emulsions, lipid droplets are evenly distributed in the surrounding aqueous phase, *i.e.* they do not exhibit creaming, coalescence, flocculation, or Ostwald ripening over a specific period. The kinetic stability is largely achieved by emulsifiers which absorb to the lipid-water interphase and lower the surface tension between the two phases. In this thesis, phospholipids were mainly used as an emulsifier (phospholipid stabilised emulsion droplet is shown in Figure 6), but surface-active proteins (*e.g.* casein) and small molecule surfactants (*e.g.* Tweens, sodium dodecyl sulphate (SDS), Brijs, Citrem) are also common [30, 50].

Different molecules partition themselves among the three regions of an emulsion (*i.e.* aqueous phase, interface, and lipid phase), according to their polarity, affinity to the interface and surface activity. Non-polar molecules are located predominantly in the oil phase, polar molecules in the aqueous phase and surface-active molecules at the interface. The composition of the emulsion droplet, and especially the interface, plays a key role in lipid oxidation because it can dictate how lipids (poly-unsaturated fatty acids, lipid hydroperoxides) will interact with components dissolved in the aqueous phase, especially prooxidants and reactive oxygen species [30].

3.2.6 Factors affecting lipid oxidation in emulsions and liposomes

The oxidation in bulk lipids (oils/fats) has been studied extensively, and nowadays there is a fairly good understanding of the factors that affect lipid oxidation in these bulk systems [51, 52]. Oxidation processes in emulsions/liposomes are more complex than in bulk lipids due to the following aspects:

- presence of the interfacial region and the aqueous phase,
- partitioning of different molecules in the different phases,
- presence of water-soluble components which are not likely to be present in bulk lipids,
- contact of the lipids with water rather than with air [30].

The nature of the interphase is perhaps the most important factor determining the oxidation rates in iron-mediated oxidation (and possibly other prooxidants as well). In the studies of Mei *et al.* it was demonstrated that iron-mediated oxidation had the lowest rates in emulsions stabilised with cationic emulsifiers and the highest rates with anionic emulsifiers (phospholipids belong to the latter category) [53, 54]. This was explained by association of the positively charged iron ions with negatively charged emulsifier molecules.

Therefore, techniques that control physical location of metals could be effective in controlling oxidation in emulsions. One way to alter the physical location of prooxidants is to introduce emulsifier micelles into the emulsion. The micelles may divert the location of prooxidants (but also

antioxidants) from the droplet interphase [55]. Another way is to introduce substances with chelating abilities (described in Section 3.3.1).

The thickness of the emulsifier also plays a key role in iron-mediated lipid oxidation. An emulsifier which would create a physical barrier between the prooxidants in aqueous phase and lipids in the core of the emulsion droplet could make it more difficult for the iron to interact with the lipids [56]. Thick emulsifiers (such as Brij 700) are not approved for food applications. Whey proteins may have sufficient thickness, but their use is limited by the pH of the aqueous phase. They are useful only when the pH is below the pI (isoelectric point) of the protein, at which the protein is cationic [12].

Hydroperoxides contained in the emulsifiers have been suggested to be another important factor in iron-mediated oxidation. Tweens, Brijs and lecithins (phospholipids) were shown to contain significant levels of endogenous hydroperoxides, which then trigger iron-mediated oxidation [55, 57, 58].

The size of emulsion droplets can vary from 0.2 μm to 100 μm in diameter meaning that the total surface area, which is in contact with the aqueous phase, varies greatly. Some studies have proposed that oxidation rates do not change dramatically with large variations in the total surface area of droplets, claiming that the surface in most cases is large enough not to limit the reaction rates [12].

The aqueous phase is a carrier for multiple components which are directly or indirectly involved in lipid oxidation, such as:

- dissolved oxygen, which is absolutely necessary for oxidation processes;
- inorganic acids and bases, which determine the pH of the system;
- prooxidants and reducing agents, which may adsorb on the interface;
- antioxidants, which partition in the three phases of emulsions, based on their polarity and type of emulsifier [59, 60];
- inorganic salts, which may adsorb on the interphase;
- other organic molecules (proteins, carbohydrates), which may form an additional layer on the surface of emulsion droplet [61, 62].

Therefore, the composition of the aqueous phase will also determine the final rates of lipid oxidation emulsions. An overview on important factors which may affect lipid oxidation in emulsions is shown in Table 4.

Table 4 Factors capable of inhibiting lipid oxidation in oil-in-water emulsions (table from [12])

Characteristic	Property	Factors
Lipid phase	Composition	• Degree of unsaturation • Prooxidant impurities (e.g., free fatty acids, hydroperoxides) • Inherent and added antioxidants
	Physical state – solid fat content and crystal properties	• Solubility, partitioning and diffusion of antioxidants and prooxidants
	Physical properties	• Rheology determines diffusion of antioxidants and prooxidants • Polarity determines partition coefficients
Aqueous phase	Composition – pH, ionic strength, solutes	• Prooxidant impurities (e.g., transition metals, photosensitizers, enzymes) • Inherent and added antioxidants • Micelles may alter location of antioxidants and prooxidants • Reducing agents that can redox cycle prooxidant metals
	Physical state – ice crystal structure and location	• Solubility, partitioning and diffusion of reactants and products
	Physical properties	• Rheology determines diffusion of antioxidants and prooxidants • Polarity determines partition coefficients
Interphase	Composition	• Anti-/prooxidant activity • Impurities (hydroperoxides)
	Thickness	• Steric hindrance of interactions between water- and oil-soluble components
	Charge	• Electrostatic attraction/repulsion of antioxidants and prooxidants
	Permeability	• Diffusion of antioxidants and prooxidants in lipid and aqueous phase
Structural	Emulsion	• Droplet concentration • Droplet size distribution (surface area and light scattering)
	Spray-dried powder	• Porosity • Exposed lipid levels • Emulsion droplet characteristics upon rehydration
	Hydrogel particles	• Hydrogel composition, structure and properties

3.3 Antioxidants

Antioxidants have been defined as "any substances that delay, prevent or remove oxidative damage to a target molecule" [63]. This definition opens for a wide range of compounds which can be defined as antioxidants. Consequently, the antioxidant activity can be manifested by various mechanisms. In relation to lipid oxidation, the following antioxidant mechanisms are recognized:

- inhibition of reactive oxygen species
- quenching free radicals
- quenching singlet oxygen
- quenching photosensitizers
- chelation of metals
- inhibition of pro-oxidative enzymes
- synergism with other antioxidants
- scavenging triplet oxygen (by reducing agents)

It is not unusual that one substance is capable of multiple antioxidant mechanisms. Which mechanism dominates often depends on the surrounding conditions. Under specific conditions, an antioxidant may increase oxidation rates, thus turning into a prooxidant.

3.3.1 Metal chelators

Metal chelators decrease the prooxidant activity of metals by

- preventing metal red-ox cycling by occupation of metal's coordination sites,
- formation of insoluble metal complexes,
- providing steric hindrance for interactions between metals and lipids or oxidation intermediates (*e.g.* hydroperoxides) [33, 64].

The most common metal chelators contain multiple carboxylic acid groups (*e.g.* (ethylenediaminetetraacetic acid (EDTA), citric acid) or phosphates (*e.g.* polyphosphates and phytates). Metals can also be controlled by metal binding proteins, such as transferrin, ferritin, phosvitin [65], lactoferrin, albumin and casein [66].

Flavonoids with the 3',4'-dihydroxy group in the B ring, the 4-carbonyl and 3-hydroxy group in the C ring, or the 4-carbonyl group in the C ring together with the 5-hydroxy group in the A ring, can also bind metal ions (depicted in Figure 7) [67].

Figure 7 Metallic ion complexation by flavonoids via the 3'4'-dihydroxy group in the B ring (**left**), 4-carbonyl and 3-hydroxy group in the C ring (**middle**) and 4-carbonyl group in the C ring together with the 5-hydroxy group in the A ring (**right**)

Most of the above mentioned compounds are soluble in aqueous solutions. Citric acid can be to some degree dissolved in oils [64].

Chelators must be ionized to be active. Therefore their activity decreases at pH values below the pK_a of their ionizable groups. Some metal chelators can increase oxidative reactions by increasing metal solubility or altering the redox potential of the metal. This is often dependent on metal-to-chelator ratio. A typical example is EDTA: It is said to be ineffective or prooxidative when EDTA-to-iron ratio is ≤ 1 and antioxidative when EDTA-to-iron ratio is > 1 [33].

3.3.2 Radical scavengers

Radicals involved in lipid oxidation are described in Section 3.2.2. Radical scavengers (HX) quench these radicals by donating hydrogen atom to them, becoming relatively stable antioxidant radicals themselves (eq. 19). Radical scavengers are believed to react mainly with peroxyl radicals ($LOO^\bullet$) due to the long life time of this radical (shown in Table 2).

$$LOO^\bullet + HX \rightarrow LOOH + X^\bullet \qquad \text{(eq. 19)}$$

Phenolic compounds, a group of approximately 8 000 compounds, all possessing one common structural feature: a phenol – an aromatic ring bearing at least one hydroxyl (–OH) group, are effective radical scavengers [68]. Phenolic compounds can be classified into three main categories according to the number of phenol units in the molecule:

a) simple phenols – one phenol unit,
b) flavonoids – two phenol units,
c) tannins – at least three phenol units.

Flavonoids and tannins are referred to as polyphenols, and all three groups can be further subdivided into numerous sub-groups based on various structural features. Phenolic compounds are widely spread throughout the plant kingdom as secondary plant metabolites. They are present either in free form or, more typically, conjugated to various molecules (acids, sugars). It is not in the scope of this thesis to provide detailed overview on the categories and occurrence of phenolic compounds, but plenty of literature exists to cover these topics – for instance [68-72].

Several phenolic compounds have been developed in the past for food, feed and cosmetic applications, known as *synthetic antioxidants*. These are butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DG), tertiary-butylhydroquinone (TBHQ), 4-hexylresorcinol and ethoxyquin (EQ). Nowadays trends in food manufacturing are to avoid using synthetic antioxidants because of healthy concerns and negative attitudes of customers to synthetic additives [73].

The number of natural (or natural identical) radical scavengers legally approved as food additives is very limited, counting only a few compounds (tocopherols, ascorbates, some carotenoids, extract of rosemary and some anthocyanins), and the legislation differs between countries and regions (*e.g.* European Union, United States, Australia, Asian countries) [74]. Therefore, identifying highly efficient and non-toxic antioxidants in the natural resources, which are suited for food/feed and cosmetic applications, is one of the goals in the research on antioxidants for the past two decades.

3.3.2.1 Hydroxycinnamic acid derivatives

Hydroxycinnamic acid derivatives (HCAD) (Figure 8) belong to the group of simple phenols. They are ubiquitous in the plant kingdom and abundant in the human diet [75, 76]. Hydroxycinnamic acid derivatives studied in **PAPER II** were: caffeic acid (3,4-dihydroxycinnamic acid, CaA), ferulic acid (4-hydroxy-3-methoxycinnamic acid, FeA) and *p*-coumaric acid (*trans*-4-hydroxycinnamic acid, CoA). Caffeic acid was also studied in **PAPER III**. The mechanisms of hydrogen donation are therefore described for this group of compounds.

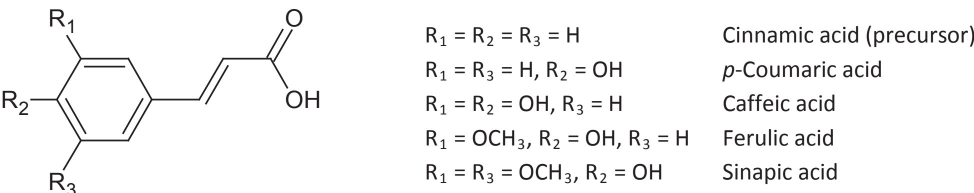

Figure 8 Hydroxycinnamic acid derivatives

The following text provides an overview on the radical scavenging mechanisms and conditions at which each mechanism prevails for HCAD. These mechanisms and similar factors are likely to be involved in radical scavenging by other phenolic compounds.

The reaction mechanisms involved in the free radical scavenging by HCAD follow two pathways:

1) *hydrogen transfer* (HT), in which –OH group donates hydrogen radical ($H^{\bullet}$), and
2) *single electron transfer* (SET), in which the –OH group donates hydrogen proton (H^{+}).

Recently it was found that hydrogen transfer is the key mechanism for reactions in non-polar media and in aqueous solutions at acidic pH for $HO^{\bullet}$, $HOO^{\bullet}$ and $LOO^{\bullet}$. The SET mechanisms is on the other hand dominant for reactions in aqueous solutions at physiological and more basic pH, and for a large variety of radicals ($N_3^{\bullet}$, $Br^{2\bullet-}$, $Cl_3COO^{\bullet}$, $SO_4^{\bullet-}$, $NO_2^{\bullet}$, DPPH radical, radical cations of dAMP and dGMP) and also $LOO^{\bullet}$ in basic solutions. For the reaction with a hydroxyl radical ($OH^{\bullet}$), a *radical adduct formation* (RAF) has been found to be significant alongside HT [77] (summarized in Table 5).

Table 5 Reaction mechanisms involved in the free radical scavenging by hydroxycinnamic acid derivatives

Reactions in non-polar medium			
Hydrogen transfer (HT):		$H_2X + LOO^{\bullet} \rightarrow HX^{\bullet} + HOOH$	(eq. 20)
Radical adduct formation (RAF):		$H_2X + LOO^{\bullet} \rightarrow [H_2X\text{–}OOH]^{\bullet}$	(eq. 21)
Reactions in aqueous medium			
Single electron transfer (SET):	a)	$HX^{-} + LOO^{\bullet} \rightarrow HX^{\bullet} + HOO^{-}$	(eq. 22)
	b)	$X^{-} + LOO^{\bullet} \rightarrow X^{\bullet} + HOO^{-}$	(eq. 23)
Hydrogen transfer (HT):		$HX^{-} + LOO^{\bullet} \rightarrow X^{-\bullet} + HOO^{-}$	(eq. 24)
Radical adduct formation (RAF):		$HX^{-} + LOO^{\bullet} \rightarrow [HX\text{–}OOH]^{-\bullet}$	(eq. 25)

The role of the side chain in the inherent antiradical activity of HCAD has not been fully established yet. Results from several papers suggest that the ethylenic side chain may not be an important factor for the antiradical activity [78, 79]. On the other hand, Amorati *et al.* proposed that it may lower the strength of the phenolic O–H bond [80]. According to León-Carmona *et al.*, in non-polar media and aqueous solutions at acidic pH, the side chain contributes to HT via its electron donating character [77].

The role of pH on the antioxidant activity of HCAD is another unresolved issue. It has been reported that the antiradical activity depends on the pH of the solution, becoming larger as the pH increases from 4 to 8, due to higher activity of the phenolate anion, compared to the neutral phenolic group [80]. However, it has been reported that that there is no relationship between the

antiradical activity and pK_a values of the compounds [78]. Recently, it was published that pH determines the radical scavenging mechanisms. Hydrogen transfer occurs at acidic pH, while SET is prevailing at physiological (7.4) and more basic pH. [77]

At the conditions where HT is the main mechanism, the key factor ruling the order of reactivity is the other group in the phenolic moiety. The presence of another OH group (catechol group, such as in caffeic acid) leads to a higher reactivity, the presence of the OCH_3 group (ferulic acid) has an intermediate effect, and no other group in the phenolic ring (*p*-coumaric acid) gives lower reactivity [77].

3.3.2.2 Tocopherols

Lipid soluble vitamin E compounds – *tocopherols* and *tocotrienols* – are well recognized for their inhibition of lipid oxidation in both foods and biological systems. Rich sources of tocopherols are for instance cereals, oilseeds, and nuts. Four analogues of tocopherol exist: α, β, γ, and δ (Figure 9), which differ in number and positions of methylene groups on the chromanol ring [68]. In this thesis, the antioxidant efficiency of α- and δ-tocopherol in LMW iron- and metHb-mediation was measured (**PAPER III**, **Additional data**).

R_1	R_2	
CH_3	CH_3	α-Tocopherol
CH_3	H	β-Tocopherol
H	CH_3	γ-Tocopherol
H	H	δ-Tocopherol

Figure 9 Chemical structure of tocopherols

The antioxidant activity of tocopherols is mainly due to their ability to donate hydrogen from their 6-phenoxyl group to lipid free radicals (eq. 26) which terminates the radical chain reactions and yields resonance stabilized tocopheroxyl radicals (Toc-O•). These may continue to eliminate lipid radicals by forming adducts with them (eq. 27) or may form non-radical products when reacting with each other (eq. 28) [30]. There is a confusion concerning their relative potency in food systems. The chemical structures of tocopherols support a hydrogen-donating power in the order $\alpha > \beta > \gamma > \delta$, but a reversed order ($\delta > \gamma \approx \beta > \alpha$) was obtained in fats and oils, and under various conditions [81]. Therefore, beside absolute chemical reactivities toward lipid radicals, other factors, such as tocopherol concentrations, temperature and light, lipid substrate, and other chemical compounds

present (metals, reductants, other radical scavengers) and reaction conditions (polarity, pH, etc.) may determine the efficacy.

$LOO^{\bullet}$ + Toc-OH $\longrightarrow$ LOOH + Toc-$O^{\bullet}$ $k = 1.85 \times 10^{6}$ s (eq. 26)

$LOO^{\bullet}$ + Toc-$O^{\bullet}$ $\longrightarrow$ adducts $k = 1.23 \times 10^{8}$ s (eq. 27)

Toc-$O^{\bullet}$ + Toc-$O^{\bullet}$ $\longrightarrow$ non-radical products $k = 1.28 \times 10^{3}$ s (eq. 28)

Under certain conditions tocopherol may act as prooxidants. The prooxidant effects have been related to high concentrations of accumulated tocopheroxyl radicals, which may react both with intact lipids (LH) or lipid hydroperoxides (reversed eq. 26, k = 10.6 s). However, rates of these reactions have been found to be much lower than the rates of adduct formation (eq. 27) and coupling of tocopheroxyl radicals (eq. 28), and are therefore considered less significant [30, 81]. Oxidation products of tocopherols (tocopheroklquinones) have been reported to be more significant in pro-oxidation reactions of tocopherols than tocopheroxyl radicals [30].

The tocopherols and tocopheroxyl radicals can reduce metal ions by donation of an electron (eq. 29 and eq. 30) and in this respect they may be considered as prooxidant synergists [81].

Toc-OH + $M^{(n+1)+}$ $\longrightarrow$ Toc-$O^{\bullet}$ + H^{+} + M^{n+} (eq. 29)

Toc-$O^{\bullet}$ + $M^{(n+1)+}$ $\longrightarrow$ TO^{+} + M^{n+} (eq. 30)

Synergistic effects have been observed for tocopherols. Three mechanisms for the synergism have been recognized: a) sparing, b) regenerating, and c) trace metal chelation.

Sparing of tocopherol occurs when another radical scavenger/singlet oxygen quencher is present alongside tocopherol and works by the same or different antioxidant mechanism.

Regenerating of tocopherol occurs in the presence of substances which are capable of donating an electron to the tocopheroxyl radical, thus re-creating the original tocopherol molecule. Biologically relevant tocopherol regenerators are ascorbic acid and glutathione.

Trace metal chelation enhances the activity of tocopherols by elimination of the reactions between tocopherol and metals (eq. 28 and eq. 29). Apart from typical metal chelators (*e.g.* citric acid), phosphatidylinositol and acidic phospholipids, some amino acids, peptides, and Maillard reaction products (melanoidins) were found to act synergistically with tocopherol due to their metal-chelating abilities [81].

3.3.2.3 Ascorbic acid and ascorbyl palmitate

Ascorbic acid (Vitamin C) has multiple functions in relation to lipid oxidation in food systems. It has been reported to act as:

- oxygen scavenger,
- scavenger of reactive oxygen species generated in the aqueous phase (*e.g.* hydroxyl radical ($HO^{\bullet}$)),
- singlet oxygen quencher,
- reducing agent,
- regenerator of tocopherol.

There is also a negative aspect of ascorbic acid action. Due to its reducing abilities, ascorbic acid can act as a prooxidant by reducing transition metals such as iron, copper and vanadium to the more catalytically active forms [82].

Ascorbyl palmitate and ascorbyl stearate are lipid soluble esters of ascorbic acid which scavenge lipid radicals in the oil phase [83]. The effect of ascorbic acid and ascorbyl palmitate on low molecular iron and metHb-mediated oxidation in emulsions and liposomes was studied in this thesis (**PAPER III**, **Additional data**).

3.3.3 Singlet oxygen quenchers

Singlet oxygen ($^1O_2$) quenching includes both *physical* and *chemical* quenching.

Physical quenching leads to deactivation of $^1O_2$ to the ground state triplet oxygen ($^3O_2$) via *energy transfer* by a ground state quencher, producing a triplet state quencher (eq. 31). The triplet state quencher dissipates the energy in the form of heat into the environment returning to the original ground state (eq. 32). Physical quenching happens when the energy level of the ground state quencher is very near or below the energy level of singlet oxygen.

Carotenoid pigments, such as carotenes (*e.g.* β-carotene, lycopene, lutein) and xanthophylls (*e.g.* isozeaxanthin, astaxanthin) represent the most active $^1O_2$ quenchers. It has been estimated that one carotenoid molecule is able to quench $\sim 1\,000$ $^1O_2$ molecules [84]. Carotenoids with ≥ 9 conjugated double bonds are good $^1O_2$ quenchers, and the efficiency improves with increasing number of the conjugated double bonds. The efficiency is also dependent on the functional groups attached to the carbon chain and the cyclopentane ring [85].

$$^{1}O_2 + {}^{1}\text{carotenoid} \longrightarrow {}^{3}\text{carotenoid} + {}^{3}O_2 \quad \text{(eq. 31)}$$

$$^{3}\text{carotenoid} \longrightarrow {}^{1}\text{carotenoid} + \textit{heat} \quad \text{(eq. 32)}$$

Chemical quenching leads to deactivation of $^{1}O_2$ to the ground state $^{3}O_2$ via *charge transfer*, and happens when the quencher has high reduction potential and low triplet state energy. Ascorbic acid, β-carotene, tocopherols, amino acids (histidine, tryptophan, cysteine and methionine), amines, sulphides, iodides and azides are examples of $^{1}O_2$ chemical quenchers. The quencher donates an electron to $^{1}O_2$ forming a singlet state charge transfer complex and then changes the complex to a triplate state complex by intersystem crossing. The triplet state charge transfer complex is then dissociated into $^{3}O_2$ and an oxidised quencher (Q') (eq. 33). This means that the quencher molecule is modified after the reaction is completed [64].

$$^{1}O_2 + {}^{1}Q \longrightarrow [O_2\text{-}Q]^{1} \longrightarrow [O_2\text{-}Q]^{3} \longrightarrow {}_{3}O^{2} + Q' \quad \text{(eq. 33)}$$

It has been reported that tocopherols are also capable of both physical and chemical quenching of singlet oxygen. Tocopherols can deactivate about 40 – 120 $^{1}O_2$ molecules, before they are destroyed which is considerably less in comparison to carotenoids [81].

3.3.3.1 Carotenoids

Carotenoids are a group of more than 600 lipid soluble compounds, which are responsible for most of the red, orange and yellow colours in the plant and animal kingdoms [86]. The main function of carotenoids is protection against photo-sensitized oxidation, *i.e.* absorption of light. In the human diet, carotenoids serve as a source of vitamin A, which is necessary for low-light and colour vision, but they are also believed to have health beneficial effects against degenerative diseases [30, 87, 88]. Carotenoids consist of two classes of compounds:

a) *Carotenes*, which are pure hydrocarbons (*e.g.* β-carotene, lycopene)

b) *Xanthophylls*, which contain oxygenated functional groups (*e.g.* astaxanthin, lutein).

The presence of an oxy (=O) and hydroxyl (–OH) group in the molecule modifies its polarity and thereby its solubility, stability, distribution in different solvents, orientation/location in phospholipid membranes, and reactivity to other compounds. The groups also enhance the antioxidant properties [30].

β-Carotene

Astaxanthin

Figure 10 Chemical structure of β-carotene and astaxanthin

As mentioned above, carotenoids are efficient quenchers of singlet oxygen and as well as of excited photosensitizers. The long conjugated double bond system of carotenoids makes them an excellent substrate for radical attack. Lipid peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals react with carotenoids at much higher rates than with unsaturated fatty acids. Therefore, carotenoids have also radicals scavenging abilities. Carotenoids are postulated to scavenge lipid radicals through addition of the radical to the conjugated system so that the resulting carbon-centred radical is stabilized by resonance. Another proposed mechanism is abstraction of hydrogen (similar to phenolic antioxidants) [30]. Synergistic effects with α-tocopherol and ascorbic acid have been reported as well [30, 89].

Under certain conditions, carotenoids can also show prooxidant effects. At low oxygen pressure (~2.0 kPa / 2% O_2) carotenoids have been shown to act as antioxidants. The antioxidant activity was however supressed at high oxygen pressure (99.99 kPa / 100% O_2) turning carotenoids into prooxidants. When carotenoids absorb blue light (400 – 500 nm) or heat, they can get excited to triplet states which might initiate or propagate lipid oxidation (eq. 13) [30].

The activity of β-carotene and astaxanthin (Figure 10) in LMW iron- and methemoglobin-mediated oxidation was studied in this thesis (**PAPER III** and **Additional data**).

3.4 Post-prandial lipid oxidation

Much attention has been paid to oxidation of food lipids during processing and storage, and positive and negative health effects of omega-3 fatty acids have therefore been mainly discussed in relation to the quality of the lipids determined prior to ingestion. Peroxidation of omega-3 fatty acids seems, however, not to end with the food production. Since the stomach is the entry organ for food, Kanner *et al.* [90] suggested that the stomach containing gastric juice may act as a "bioreactor" promoting peroxidation of dietary lipids after digestion, which is called *post-prandial* lipid oxidation. It has also been postulated that peroxidation of dietary lipids may continue in subsequent parts of the gastrointestinal (GI) tract – the *s*mall and large intestine [11].

Varying health effects reported in clinical studies could therefore be to some degree attributed to oxidation occurring after ingestion of the lipids, and the overall health effect might be governed by the oxidative state of the fatty acids right before absorption by the GI tract [10].

Secondary lipid oxidation products, such as aldehydes, malondialdehyde and 4-hydroxy-2-*trans* nonenal, have been reported to be cytotoxic [9, 27, 91, 92]. Consumption of oxidised PUFA or oxidation of PUFA after ingestion would expose the cells of the GI tract to the full force of these toxic agents, forming a risk factor for cancer development primarily in the GI tract. [11]

3.4.1 Gastric juice

Gastric juice is a complex solution of components secreted by the parietal cells of the stomach. The component governing the level of acidity (pH 1 – 4) is hydrochloric acid (up to 0.1 M). Other important component are inorganic salts (KCl, NaCl, $CaCl_2$, bicarbonates, and calcium and magnesium phosphates), a series of digestive enzymes (pepsin, gastricin, gelatinase and other gastric lipases and amylases), proteins (gastroferrin, glutathione, albumins and globulins) and glycoproteins (mucin, intrinsic factor) and various hydrocarbons [93]. Gastric juice also contains saliva which enters the stomach with the ingested food.

The use of authentic human gastric juice for *in vitro* digestion models, rather than animal analogues or artificial formulations has recently been highlighted, since the human digestive juices (both gastric and duodenal juice) contain enzymes of various isoforms that may differ from those obtained from animals when it comes to both specificity and activity. In addition, the physiological combination and ratios of gastric juice constituents are unlikely to be achieved in artificial gastric juice formulations [94].

Gastric mucosa also secrets the tripeptide *glutathione*. Among other functions, glutathione works as an endogenous antioxidant (reducing agent) directly participating in the neutralization of

free radicals and reactive oxygen species [95], as well as it may maintain exogenous antioxidants such as vitamin C (ascorbic acid) and vitamin E (tocopherols) in their reduced, *i.e.* active, forms [96].

3.4.2 Oxidation of lipids in stomach

The stomach is an entry organ for food and can be seen as an intermediate station, sort of a *dynamic bioreactor*, before the food is moved further to the gastrointestinal tract – intestines and metabolised.

Hydrolysis of the lipid molecules by gastric and lingual lipases occurs to a certain degree (up to 30%) already in the stomach, but lipids are mainly metabolized by intestinal lipases and transferred into the blood stream from the intestines [97]. It is therefore reasonable to assume that during the time the LC omega-3 fatty acids from food, both hydrolysed and non-hydrolysed, are retained it the stomach, they can oxidatively deteriorate, *i.e.* undergo *post-prandial* oxidation.

For LC omega-3 fatty acids to oxidise in the gastric juice, oxygen needs to be supplied to the closed stomach. It has been proposed that saliva, masticated food, liquids and swallowed air bring enough oxygen into the stomach, which can then facilitate post-prandial oxidation of lilpids [90].

However, studies evaluating and quantifying post-prandial oxidation of sensitive LC omega-3 PUFA are scarce. The reason for this lies in difficulties with *in vitro* simulation of the complex physiological processes occurring in the gastrointestinal tract after the food/lipids are ingested, including modelling of the compositionally complex gastric and duodenal juices [98]. To date, no published study attempted to investigate post-prandial lipid oxidation *in vitro*, *i.e.* using humans as experimental models. However, a recent study by Chen et al. [99] attempted to develop a simple device for routine *in vitro* gastric digestion investigations.

Lipids are usually not the only nutrients entering the stomach. Food also frequently contains prooxidants and antioxidants, which implies that both oxidation mediated by prooxidants and antioxidative reactions facilitated by dietary antioxidants may occur during post-prandial oxidation of lipids. The idea that the gastrointestinal tract could be the location for the protective activity of antioxidants, such as tocopherols, ascorbic acids, carotenoids and phenolic compounds, was initially presented by Halliwell *et al.* [11]

Relevant studies on post-prandial lipid oxidation are mentioned here and a few more are mentioned in **PAPER IV**:

A recent study of Larsson *et al.* [100] investigated oxidation of cod liver oil during a gastrointestinal static *in vitro* digestion in artificial gastric and duodenal juices. The authors concluded that fresh marine oils, with and without commercial levels of α-tocopherol, can give rise to toxic oxidation

products during the gastrointestinal passage, and that relatively small difference in the initial oil quality may be enlarged during digestion.

In the study of Kenmogne-Domguia *et al.* [101], protein and phospholipid-stabilised emulsions containing tuna oil oxidised during *in vitro* digestion in the gastric step and also during the intestinal phase (measured by quantification of malondialdehyde, 4-hydroxy-2-hexenal and 4-hydroxy-2-nonenal, and head space oxygen). Furthermore, the endogenous tocopherols were consumed throughout the digestion.

Lapidot *et al.* [102] studied how iron (Fe^{3+}), myoglobin and dietary antioxidants (ascorbic acid, polyphenols) affect lipid oxidation in both human and simulated gastric juice. The oxidizable fatty substrate was grilled red turkey muscle and linoleic acid emulsion. The authors concluded that peroxidation of the meat, and iron- and myoglobin-mediated oxidation in the emulsion can be inhibited by high concentrations of ascorbic acid and polyphenols.

In the studies of Gorelik *et al.* [103, 104] it has been observed that the consumption of meal rich in oxidisable fat together with a rich source of antioxidants, red wine concentrate, reduced the absorption of lipid hydroperoxides and their secondary oxidation products (MDA) into the plasma, as a consequence of the antioxidative effect of the red wine polyphenols.

Because of small number of studies addressing *post-prandial* oxidation of lipids, there is not enough evidence to draw solid conclusions on this topic, and especially on post-prandial oxidation of marine lipids. Therefore, in **PAPER IV**, oxidation of marine lipids in liposomes and emulsion in the presence of authentic human gastric juice is investigated, and the effect of several antioxidant rich beverages is evaluated.

3.5 Measurement of lipid oxidation

As described in Section 3.2.2, lipid oxidation is a complex set of radical reactions which give an enormous variety of oxidation products, and where the essential reactant is oxygen (O_2) [32] (summarized in Figure 11). Numerous methods have been developed for the measurement of lipid oxidation – reviewed for example by Barriuos *et al.* [105] or Shahidi *et al.* [106]. The majority of these methods is based on measurement of one specific or a set of lipid oxidation products.

A drawback in the determination of lipid oxidation products is that these products are not stable and their concentrations vary with time. In classical autoxidation, the concentration of lipid hydroperoxides (LOOH) increases during the early phase of oxidation, but is decreased in later stages due to the decomposition of the LOOH into secondary oxidation products. On the contrary, secondary oxidation products (predominantly volatiles) are absent in the early stages of lipid oxidation and accumulate after the LOOH are broken down [29]. Some of the secondary oxidation products may be further broken down or reacted into tertiary oxidation products. Because of their volatile nature, some of these products may even evade determination. Once prooxidants, antioxidants and reaction oxygen species are involved in the oxidation processes, the overall spectrum and ratios of the oxidation products may change significantly. In addition, oxidation of LC omega-3 PUFA gives a much broader spectrum of volatiles than less unsaturated fatty acids which further complicate their assessment [29, 32].

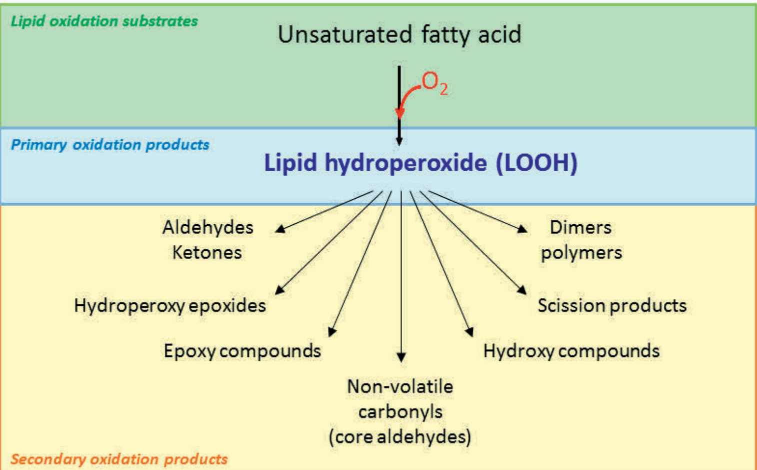

Figure 11 Simplified overview on substrates, and primary and secondary products in lipid oxidation (adapted from [29])

Therefore, measuring a few selected oxidation products (lipid oxidation markers) may give incomplete or even misleading information about the lipid oxidation status. It has been suggested to use several methods simultaneously to characterize lipid oxidation status. The research focus should be on new and advanced techniques, such as electron paramagnetic resonance (EPR), nucleic magnetic resonance (NMR) or Raman spectroscopy [105].

3.5.1 Oxygen uptake measurement in lipid oxidation studies

The rate of oxygen consumption by unsaturated fatty acids directly reflects the rate of lipid oxidation [39], assuming that other (side) reactions consuming oxygen are eliminated or not present, and that the oxygen transport in the lipid system is not limited. Mozuraityte *et al.* [39] showed that the increase in peroxides (PV) and TBARS was directly correlated with consumption of dissolved oxygen in marine liposome dispersions (Figure 12) and that iron-mediated oxidation resulted in the formation of new lipid hydroperoxides, *i.e.* peroxidation.

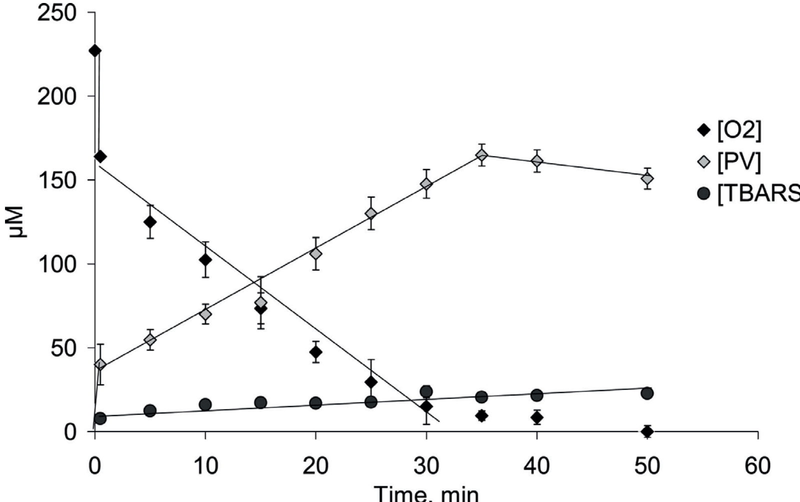

Figure 12 Changes in the concentration of dissolved oxygen [O2], peroxides [PV], and thiobarbituric acid reactive substances [TBARS] as a function of time in 0.9% liposome dispersion (pH 5.5) made of cod roe phospholipids. The oxidation was mediated by 7.5 µM Fe^{2+} added to the dispersion at t = 0 min (figure from [39]).

Measurement of changes in the lipid oxidation substrate – *oxygen* – was therefore used to evaluate LMW iron- and methemoglobin-mediated lipid oxidation in marine emulsions and liposomes in this thesis. In comparison to the measurement of lipid oxidation products, this approach

has been distinctively less common in the reported lipid oxidation studies during the past 20 years. A brief overview on the use of the oxygen uptake method in relation to lipid oxidation studies is presented here:

- Mozuraityte *et al.* studied free iron (Fe^{2+}/Fe^{3+})-mediated oxidation in marine liposomes and the effect of phospholipid and iron concentration, temperature, pH and salts on free iron-mediated oxidation [39, 107].
- Carvajal *et al.* measured oxygen uptake kinetics of cod and bovine hemoglobin in marine liposomes, and investigated the effect of pH, temperature, EDTA, astaxanthin and α-tocopherol on the prooxidant activity of the two hemoglobins [108].
- Niki *et al.* investigated synergistic interactions between tocopherol and ascorbic acid in methyl linoleate solutions [109].
- Zennaro *et al.* developed a method for determination of peroxyl radical trapping capacity and peroxyl radical trapping efficiency of water soluble and H-atom donor antioxidants in a micelle system, based on rigorous treatment of the oxygen uptake kinetic data [110].
- Roginsky *et al.* measured the capability of metmyoglobin and hemin to catalyse lipid oxidation in methyl linoleate in micellar solutions. The authors determined the rates of free radical generation from the rates of oxygen consumption, and proposed a kinetic model for these processes. [111]
- Fukuzawa *et al.* studied peroxidation of egg yolk phosphatidylcholine liposomes induced by ascorbic acid and Fe^{2+} using amongst other methods oxygen consumption. The authors investigated interactions of ascorbic acid and Fe^{2+} with fatty acids and lipid hydroperoxides on the phospholipids [112].
- Kristensen *et al.* evaluated the pro-oxidative activity of heat-denatured metmyoglobin in linoleic acid emulsions using amongst other methods oxygen uptake [113].
- Skibsted *et al.* studied radical scavenging abilities of carotenoids (astaxanthin, β-carotene, canthaxanthin, and zeaxanthin) in peroxidising of methyl esters of unsaturated fatty acids mediated by metmyoglobin and a synthetic free-radical initiator in a homogenous chloroform solution [114].

These diverse studies demonstrate that oxygen uptake method have a broad spectrum of use in lipid oxidation studies. The rare utilization of this method suggests that oxygen uptake is currently an underrated and neglected tool for investigation of rates, factors and mechanisms in lipid oxidation.

4 Experimental work

The experimental work in this thesis, including detailed descriptions of material and methods, was carried out as described in **PAPER I – IV** and in Section 5 (**Additional data**).

Measurement of dissolved oxygen concentration (oxygen uptake) was used in this thesis as the principal method for assessment of lipid oxidation. The oxygen uptake method is therefore more closely described in Section 4.2. In addition to the measurement of the dissolved oxygen concentration a number of other analytical techniques were used in this work for

- characterization of lipid composition and purity,
- determination of oxidative status of lipids,
- preparation and characterization of emulsions/liposomes, and
- characterization of the aqueous phase.

A comprehensive overview on the methods/techniques used in the thesis is given in Table 6.

4.1 Work overview

The experimental work is presented in four papers. In addition, previously unpublished research carried out in the frame of this thesis is presented (Section 5). A schematic overview on the work in this thesis and connections between the papers and the additional data is shown in Figure 13.

PAPER I studies low molecular weight (LMW) iron-mediated lipid oxidation in marine emulsions and liposome dispersions, and characterizes the impact of different factors on the rate of LMW iron-mediated oxidation in the two lipid systems. The outcomes, along with a study on methemoglobin (metHb)-mediated lipid oxidation (presented in **Additional data**), are used in **PAPER II** and **PAPER III**, which evaluate the effects of different dietary antioxidants on LMW-iron and metHb-mediated oxidation in the emulsions and liposome dispersions. Finally, **PAPER IV** builds upon the contributions from **PAPER I, II**, and **III** and **Additional data** and evaluates oxidation of marine lipids in emulsions and liposomes in the environment of authentic human gastric juice. The effect of LMW iron, metHb and antioxidant rich beverages on lipid oxidation in the human gastric juice is also evaluated.

A schematic overview summarizing the factors, antioxidants, and properties of lipid droplets studied in this thesis in relation to the prooxidant activity of LMW iron and methemoglobin in emulsions and liposome dispersion is shown in Figure 14.

Measurement of dissolved oxygen concentration

PAPER I
Iron
Liposomes
Emulsions
pH
Factors

PAPER II
Iron
MetHb
Liposomes
Phenolic aox
pH
Aox activity tests

Additional data
MetHb
Liposomes
EDTA
Hydrophilic aox
Lipophilic aox

PAPER III
Iron
Emulsion
Chelators
Lipophilic aox
Ascorbic acid
Caffeic acid

PAPER IV
Iron
MetHb
Emulsion
Aox in beverages
Gastric juice (pH)

Figure 13 A schematic overview and connections between the work curried out in this thesis (aox = antioxidants; metHb = methemoglobin)

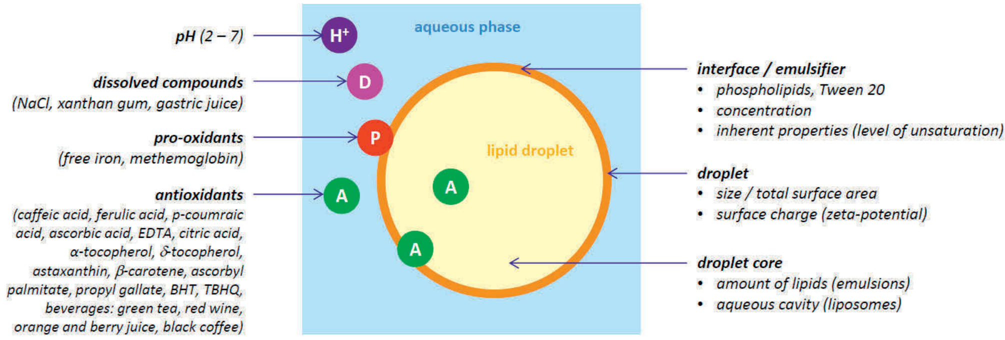

Figure 14 A schematic overview over the factors, antioxidants and properties of lipid droplets studied in this thesis in relation to free iron- and methemoglobin-mediated oxidation in emulsions and liposome dispersions

4.2 Measurement of dissolved oxygen concentration

The concentration of dissolved oxygen in the liposome dispersions and emulsions was measured by the *Clark type polarographic oxygen sensor* (oxygen electrode) which is the pivotal component of the *Oxygraph* system (Hansatech Instruments Ltd., Norfolk, UK).

The Oxygraph system (Figure 15) consists of a cell unit mounted on a control unit. The electrode disk forms the floor of the reaction chamber in the cell unit, into which liposome dispersion/emulsion (or any other liquid) is transferred – the reaction volume is optional in the range 0.2 – 2.5 mL. A magnet inserted into the cell maintains an equal distribution of oxygen throughout the volume; the stirring rate can be set between 0 – 900 rpm (in % steps). A plunger is designed so that it minimises oxygen (O_2) diffusion from the air. A capillary channel in the plunger enables injection of reactants during oxygen uptake measurements. The reaction cell is water jacketed and the jacket is connected to a circulating water bath. This enables performing experiments at a stable and optional temperature (the equipment tolerates 4 – 60 °C).

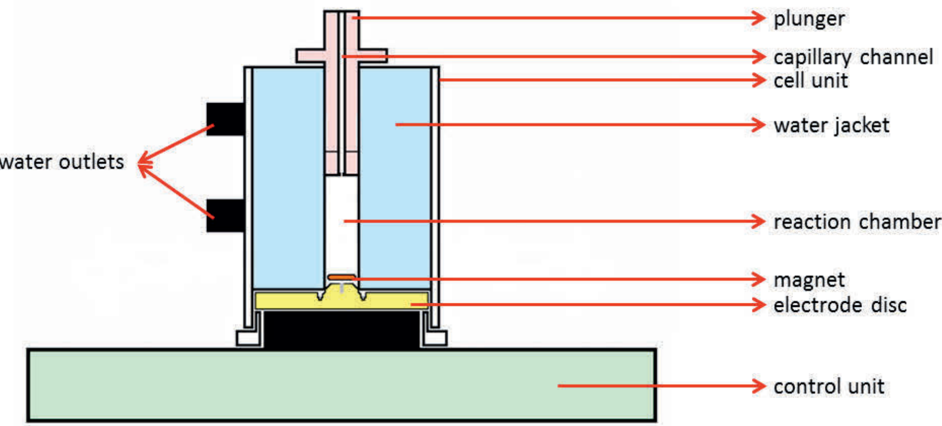

Figure 15 A schematic figure of the Oxygraph unit with description of the unit's parts

The *oxygen electrode disc* (Figure 16) consists of two electrodes – a platinum (Pt) cathode and a silver (Ag) anode – embedded in a resin. A bridge between the electrodes is established by a spacer paper saturated with an electrolyte solution (3 M KCl). The paper is held in place by a polytetrafluorethylene (PTFE/Teflon) membrane, which is permeable for O_2, and by a rubber O-ring. Another O-ring located between the electrode disc and the cell unit is providing a waterproof connection, thus preventing leakage of the electrolyte solution.

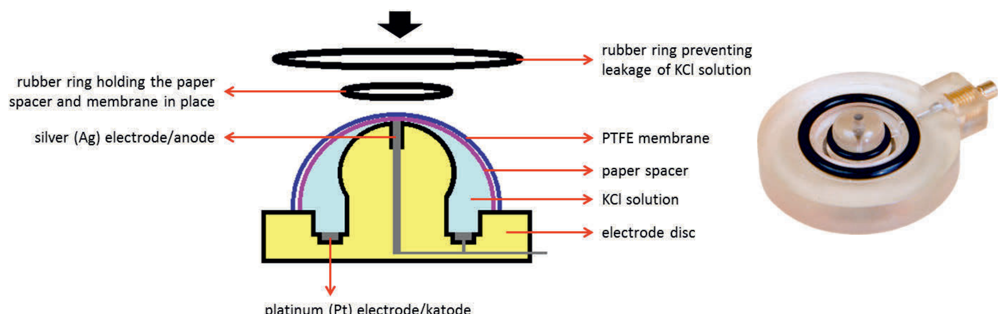

Figure 16 Left: A schematic figure of the Clark polarographic oxygen sensor (oxygen electrode), including location of silver and platinum electrode, PTFE membrane, paper spacer, KCl solution, and rubber rings; **Right:** Clark polarographic electrode disc (figure retrieved from: http://hansatech-instruments.com/products/introduction-to-oxygen-measurements/oxygen-electrode-discs/s1-oxygen-electrode-disc/)

The red-ox reactions occurring on the oxygen electrode are depicted in Figure 17. When a stable polarizing voltage from the control unit is applied the Ag-electrode becomes positive (anode) and the Pt-electrode becomes negative (cathode) and polarised (*i.e.* it adopts the externally applied potential). When the potential is increased to 700 mV, O_2 which diffuses through the membrane, is reduced at the Pt surface (initially to H_2O_2 which is then reduced to OH^-) by electrons (e^-) released from the oxidised Ag-electrode. Oxygen therefore acts as an electron acceptor in order to discharge the applied polarity. The current (e^-) flows through the circuit which is completed by KCl solution, and silver chloride (AgCl) is deposited on the anode. The generated current bears a direct (stoichiometric) relationship to the reduced oxygen and is converted to a digital signal and recorded by the control unit.

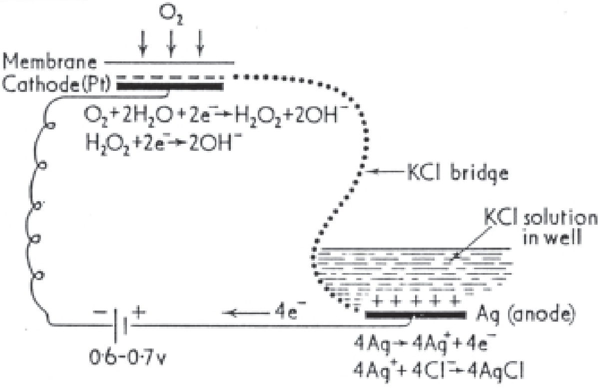

Figure 17 Principle of oxygen measurement by the Clark polarographic oxygen electrode

The digital signal is then processed by the software (Oxyg32) giving oxygen concentration, and the oxygen concentrations (μM dissolved O_2) is plotted over time (min), giving oxygen consumption curves. Examples of such curves and the quantification of oxygen uptake rates (OUR) from the curves are given in **PAPER I – IV**. More details on the Oxygraph system and the measurement of dissolved oxygen in the aqueous phase can be found in the instrument's manuals and a technical specifications sheet [115-117].

Prior to conducting a set of experiments, the chambers were thoroughly cleaned and the electrode discs were thoroughly polished to assure maximum and consistent sensitivity. The electrodes were calibrated against air saturated distilled water and air depleted distilled water at a specific temperature (most experiments were performed at 30 °C; the temperature was measured directly in the cells by a temperature sensor). The oxygen depletion was achieved by addition of sodium dithionite ($Na_2S_2O_4$) directly to the volume of water in the cell. Sodium dithionite reacts with O_2 dissolved in aqueous solutions (eq. 34). The electrodes were recalibrated after each set of experiments (typically 10 – 15 runs). Three oxygraphic cells were run simultaneously for each experiment.

$$Na_2S_2O_4 + \mathbf{O_2} + H_2O \longrightarrow NaHSO_4 + NaHSO_3 \qquad \text{(eq. 34)}$$

The measurement of oxygen concentration by the Clark type polarographic oxygen sensor has a resolution of 0.0003% [117]. The repeatability of single oxygen concentration measurements was determined using distilled water saturated with air as a stable environment. The average value of 61 consecutive measurements (1 min of oxygen concentration recording) gave a relative standard deviation of 0.07%. These data show a very low error in the determination of oxygen concentration.

The repeatability of oxygen uptake measurements is highly dependent on the calibration of the electrodes and their sensitivity, oxidative stability of the lipid system which is being measured over time, and the skilfulness of the operator in terms of transfer of the reaction volumes, injection of reactants, and thorough cleaning of the cells in between runs. Nevertheless, ten randomly chosen repetitive measurements (n = 3 – 6) of the background OUR in freshly made liposome dispersions or emulsions, using the same electrode and performed within a 1 h period, gave relative standard deviations between 5 – 18 % with an average of 9.4%.

To minimize oxidation of the emulsions and liposome dispersions over time, the dispersions were kept on ice. In case of longer periods in between runs (> 1 h), the liposome dispersions/emulsions were saturated with N_2 to minimize the content of the dissolved oxygen, and kept in a closed flask on ice.

Pro- and antioxidants were added to the emulsions and liposome dispersions dissolved in ethanol, distilled water or MES solution. The addition of 5 – 100 μL of these carriers into the liposome dispersions/emulsions did not have significant effects on the oxygen uptake rates.

Addition of pro- and antioxidants into the carrier of liposome vesicles/emulsion droplets, *i.e.* MES solution or distilled water, did not lead to changes in the oxygen consumption either, which confirmed that oxygen uptake in the emulsions and liposomes was caused by the fatty acids (data not shown).

Distilled water saturated with air contains 230 μM O_2 (at 30 °C) [115] and similar values were measured in liposome dispersions and emulsions saturated with air. After the addition of pro- and antioxidants, a fast depletion of the dissolved O_2 was observed in the closed system, eventually leading to a complete depletion of O_2. In some experiments, it was desired to follow oxidation longer, *i.e.* "beyond" the O_2 depletion point, to investigate the maximum capacity of some pro- and antioxidants. To be able to follow the oxygen consumption further, one or several air re-saturation steps were performed without interruption of the recording of O_2 concentration. The cell was opened when the concentration of the dissolved O_2 reached almost zero, air was quickly infused into the system until the O_2 concentration reached a saturated level, and the cell was closed again. After the curve was recorded, the periods with a rapid increase in O_2 concentration due to the re-saturation (usually lasting less than 1 min) were removed from the recordings and the remaining fragments of the curve were connected. The resulting "processed" curve represents a sum of μM dissolved oxygen (ΣO_2) (y-axis) consumed over time (x-axis). An example of the re-saturation steps in the oxygen uptake curve recording and the processed curve composed of the curve fragments is shown in Figure 18. Experiments in which the O_2 recordings were prolonged this way were mainly performed with methemoglobin (shown in 5.1) and ascorbic acid.

4.3 pH verification

The pH of solutions, liposome dispersions and emulsions was measured by a TIM900 Titrator manager (TitraLab, Radiometer Analytical, Copenhagen, Denmark) coupled with a combination glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, USA), which was calibrated daily against standard buffer solutions, pH 4.0 and 7.0, at 22 °C. The pH in the liposome dispersions and emulsions was verified both before and after the oxygraphic experiments.

4.4 Statistical analysis

Microsoft Excel was used for calculations and data processing. The statistical program Minitab® was used for statistical analyses. To assess significant differences, the data were subjected to analysis

of variance (one-way ANOVA), followed by a Tukey test. The level of significance was set to 95% (p = 0.05).

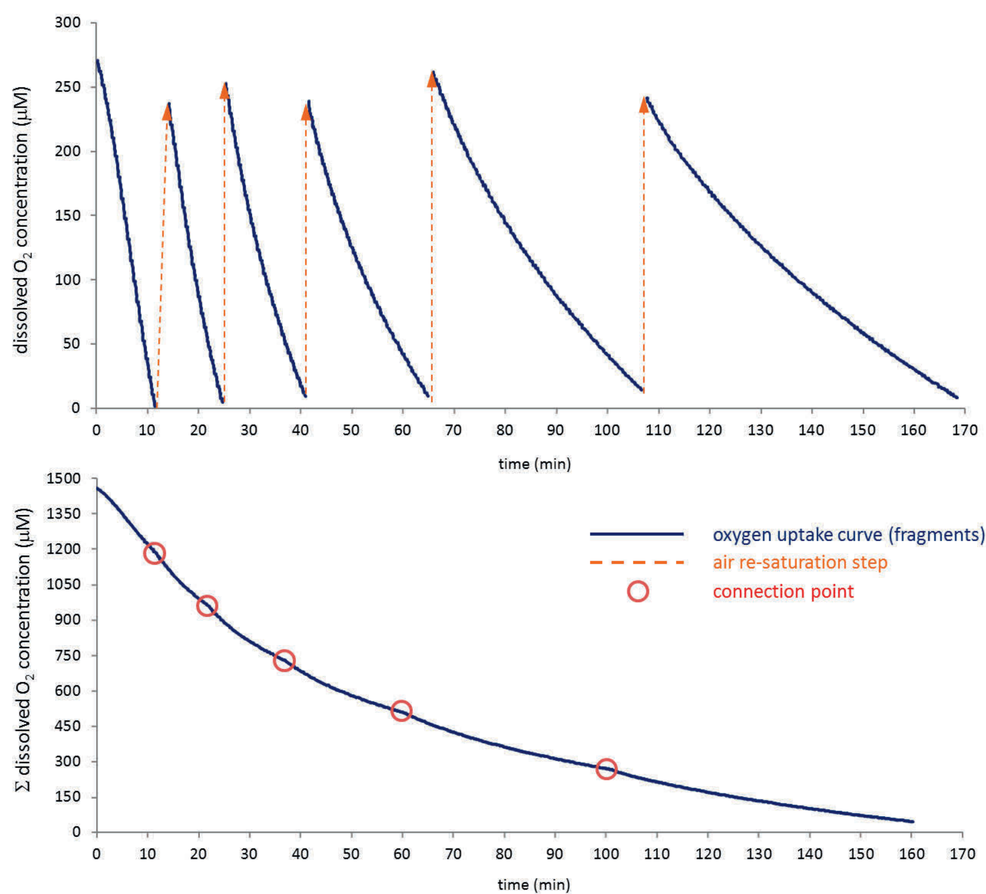

Figure 18 An oxygen uptake curve recording with air re-saturation steps (upper plot) and a processed curve composed of the curve fragments after the removal of the re-saturation periods (lower plot).

Table 6 Methods and techniques used in the PhD work

Technique	Paper I	Paper II	Paper III	Paper IV	Additional data	According to
Oxygen uptake	✓	✓	✓	✓	✓	Mozuraityte *et al.* [39, 107, 118] + instrument manual [115, 116]
Probe sonication	✓	✓	×	✓	✓	Mozuraityte *et al.* [39, 107, 118]
Emulsification with Ultra Turrax	✓	×	✓	✓	×	Instrument manual
Peroxide value (iodometric titration)	✓	×	✓	✓	×	AOCS Cd 8b-90 [119] applied in TTIP02-01AFD/2002-06A [120]
Peroxide value (ferro-thiocyanate UV-VIS)	✓	✓	✓	✓	×	Stine *et al.* [121] + modifications [122-125] + PAPER I
TBARS in oil	✓	✓	✓	✓	×	Ke & Woyewoda [126]
TBARS in emulsions/liposomes	×	×	×	✓	×	McDonald & Hultin [127]
Anisidine value	✓	×	✓	✓	×	AOCS Cd 18-90 [128]
Lipid classes (Iatroscan TLC-FID)	✓	✓	✓	✓	×	Fraser *et al.* [129], Rainuzzo *et al.* [130]
Phospholipid classes (^{31}P NMR)	×	✓	×	×	×	Internal protocol – NMR laboratories at NTNU
Phospholipid classes (HPLC-Corona CAD)	×	×	✓	✓	×	ESA application note 70-6506 [131] + PAPER III, IV
Total carotenoids	✓	×	✓	×	×	Tolasa *et al.* [132]
Methylation of fatty acids	✓	✓	✓	✓	×	AOCS Ce 2-66 [133]
Fatty acid classes (GC-FID)	✓	✓	✓	✓	×	Dauksas *et al.* [134]
Bligh & Dyer oil extraction	✓	✓	✓	✓	✓	Bligh & Dyer [135]
Acetone precipitation of phospholipids	✓	✓	✓	✓	✓	Kates [136, 137]
Oil polishing	✓	×	✓	✓	×	Crexi *et al.* [138]
Droplet charge (zeta-potential)	✓	×	✓	×	✓	Instrument manual
Average droplet size diameter	✓	×	✓	×	×	Instrument manual
Droplet size distribution	✓	×	✓	×	×	Instrument manual
Chloride titration	✓	×	×	×	×	TTEP01-04AFD/2001-05A [139]
pH determination	✓	✓	✓	✓	✓	Instrument manual
Gastric acidity	×	×	×	✓	×	TTEP01-01PHR/2001-10A [140]
Moisture content (Karl-Fisher titration)	×	×	×	✓	×	T550VKF041 [141]
Folin-Ciocalteu assay	×	✓	×	×	×	Singleton *et al.* [142] + modifications [143, 144]
FRAP assay	×	✓	×	×	×	Benzie & Strain [145] + modifications [143]
DPPH assay	×	✓	×	×	×	Brand-Williams *et al.* [146] + modifications [143, 147, 148]
ABTS assay	×	✓	×	×	×	Nenadis *et al.* [149] + PAPER II

Table 7 Characterization of lipids used in the thesis in terms of oxidation status (PV, AV, TBARS), carotenoid content and lipid classes and fatty acid composition

	Phospholipids							Herring oil		Cod liver oil
Types of lipids and origin	Herring Raw roe	Herring Washed roe	Cod Raw roe (1)	Cod Raw roe (2)	Cod Washed roe	Soy	Bacteria	Polished (1)	Polished (2)	Refined
Part of the thesis	PAPER I, III, IV	PAPER I	PAPER I	PAPER II Additional data	PAPER I Additional data	PAPER I	PAPER I	PAPER I, III	PAPER IV	PAPER I
PV (mmol LOOH/kg)	37.6 ± 1.1 42.7 ± 2.1	35.0 ± 2.0	15.2 ± 1.1	3.3 ± 0.6	9.7 ± 0.8	8.1 ± 1.4	1.6 ± 0.2	7.47 ± 0.09	4.3 ± 0.1	7.3 ± 0.4
AV	16.5 ± 0.8	20.3 ± 0.6	NA	NA	16.4 ± 1.2	NA	NA	1.61 ± 0.12	NA	NA
TBARS (mmol/kg)	1.1 ± 0.3 1.08 ± 0.13	1.0 ± 0.1	NA	2.4 ± 0.2	0.47 ± 0.5	NA	NA	0.47 ± 0.03	0.46 ± 0.02	NA
Total carotenoids (mg/kg)	10.8 ± 0.7	9.1 ± 0.8	7.9 ± 0.7	NA	14.7 ± 1.1	NA	NA	1.80 ± 0.09	NA	NA
Lipid classes (%)										
triacylglycerols (TAG)	< LOD	< LOD	< LOD	< LOD	< 0.8	< LOD	< LOD	98.9	97.6	NA
cholesterol	< 0.8	< 0.8	< 0.8	1.0	< 0.8	< LOD	< LOD	< 0.8	< 0.8	NA
unspecified	< 0.8	< 0.8	1.1	1.4	< 0.8	< 0.8	< 0.8	< 0.8	0.8	NA
phospholipids (PL)	99.5	99.7	98.2	97.9	98.3	99.5	99.5	< 0.8	1.4	NA
Fatty acid profile (%)										
saturated	27.8	29.2	33.3	29.7	26.6	25.9	50.1	22.4	22.4	18.0
mono-unsat.	16.7	16.9	24.6	25.0	23.1	8.7	46.9*	59.1	58.8	51.1
of which CET	2.9	3.5	0.5	NA	2.2	9.2	< 0.2	38.1	38.3	16.1
ERU	0.5	0.6	2.3	NA	< 0.2	1.4	< 0.2	2.2	2.2	< 0.2
GAD	9.2	11.5	17.1	12.4	18.6	1.3	< 0.2	25.8	25.9	24.0
OLE	35.2	33.7	44.9	71.9	55.4	76.2	0.8	19.7	19.8	36.8
di-unsat.	1.0	0.9	1.4	0.4	0.7	56.7	< 0.2	1.7	1.7	1.7
poly-unsat. (db ≥ 3)	54.5	53.1	40.6	44.9	46.0	8.7	2.9**	16.8	16.0	29.2
of which EPA	23.4	24.8	32.5	30.8	26.6	1.7	1.1	40.5	40.5	30.6
DHA	69.0	65.7	62.3	65.9	66.3	2.8	< LOD	40.0	40.0	48.4
DPA	2.0	2.9	2.3	2.7	1.8	< 0.2	< LOD	4.3	4.3	3.7

NA = not analysed; LOD = limit of detection; db = double bond; The values are given as a mean value ± standard error or standard deviation or with a coefficient of variation of *n* measurements (specified in **PAPER I – IV** in the analyses descriptions); CET = cetoleic acid (C22:1 n11), ERU = erucic acid (C22:1 n9), GAD = gadoleic acid (C20:1 n11); OLE = oleic acid (C18:1 n9), EPA = eicosapentaenoic acid (C20:5 n3), DHA = docosahexaenoic acid (C22:6 n3), DPA = docosapentaenoic acid (C22:5 n3), unsat. = unsaturated; * the predominant mono-unsaturated fatty acid in bacterial phospholipids is palmitoleic acid (C16:1 n7 cis); ** the predominant poly-unsaturated fatty acid in bacterial phospholipids is eicosatrienoic acid (C20:3 n3)

Table 8 Liposome dispersions and emulsions used in this thesis

	Total lipids (w/v)	Interfacial bilayer / Emulsifier (w/w lipid base)	Oil	Aqueous phase	pH	Zeta-potential at pH 5.5 (mV)	Average droplet diameter (µm)	Chloride content (µmol/g PL)
				PAPER I				
Liposomes	0.6%	raw cod roe PL (1)	–	5 mM MES	2.0 – 7.0			
	0.9%	bacterial PL	–	5 mM MES	5.5			
	1.5%	raw cod role PL (1)	–	5 mM MES	5.5	–37.2 ± 0.5		< LOD
	1.5%	washed cod roe PL	–	5 mM MES	5.5	–8.1 ± 0.6	0.097 ± 0.003	152 ± 2
	1.5%	raw herring roe PL	–	5 mM MES	5.5	–26.2 ± 4.7		< LOD
	1.5%	washed herring roe PL	–	5 mM MES	5.5	–50.4 ± 1.7		35 ± 2
Emulsion	0.5%	bacterial PL (3%)	cod liver	5 mM MES	5.5			
	0.6%	bacterial PL (3%)	cod liver	5 mM MES	3.0 – 7.0			
	0.6%	bacterial PL (0.5 – 10.0%)	cod liver	5 mM MES	5.5			
	0.6%	soy PL (1.25 – 15.0%)	cod liver	5 mM MES	5.5			
	1.5%	Tween 20 (2.5 – 15.0%)	cod liver	5 mM MES	5.5			
	5.0%	raw herring roe PL (9%)	herring (1)	distilled water	2.5 – 7.0			
	10.0%	raw herring roe PL (9%)	herring (1)	distilled water	3.5 – 4.0	–13 ± 3	10.9 ± 0.1	
	10.0%	soy PL (9%)	herring (1)	distilled water	5.1 – 5.7			
				PAPER II				
Liposomes	1.5%	raw cod roe PL (2)	–	5 mM MES	5.5, 3.0	–33.9 ± 0.6	0.087 ± 0.003	
				PAPER III				
Emulsion	10.0%	raw herring roe PL	herring (1)	distilled water	3.5 – 5.5	–13 ± 3	10.9 ± 0.1	

Table 8 Liposome dispersions and emulsions used in this thesis (continuation)

	Total lipids (w/v)	Interfacial bilayer / Emulsifier (w/w lipid base)	Oil	Aqueous phase	pH	Zeta-potential at pH 5.5 (mV)	Average droplet diameter (µm)	Chloride content (µmol/g PL)
PAPER IV								
Liposomes	2.5%	raw herring roe PL	–	5 mM MES + 0.11 M HCl	4.0			
	2.5%	raw herring roe PL	–	5 mM MES + gastric juice	4.0			
Emulsion	10.0%	raw herring roe PL	herring (2)	distilled water + 0.11 M HCl	4.0			
	10.0%	raw herring roe PL	herring (2)	distilled water + gastric juice	4.0			
Additional data								
Liposomes	1.5%	raw cod roe PL (2)	–	5 mM MES	5.5	–33.9 ± 0.6	0.087 ± 0.003	
	1.5%	washed cod roe PL	–	5 mM MES	5.5	–8.1 ± 0.6	0.097 ± 0.003	152 ± 2

HCl = hydrochloric acid, LOD = limit of detection, MES = 2-(*N*-morpholino)ethanesulfonic acid, PL = phospholipids

5 Additional data

The main results and outcomes of this thesis, including in-depth discussions, are reported in **PAPER I – IV**. Supportive data, such as characterization of the lipids, emulsions and liposomes, and preparation of the liposome and emulsion systems, are also reported in the papers.

For a more comprehensive overview, the oxidative status (PV, TBARS, AV) and composition (fatty acid classes, lipid classes, carotenoid content) of all the lipids, *i.e.* phospholipids and fish oils, used in this thesis is given in Table 7. An overview over all the liposome dispersions and emulsions used in this thesis is given in Table 8.

This section contains previously unpublished research. The additional data include studies on:

- Methemoglobin-mediated lipid oxidation (Section 5.1)
- Endogenous metals in liposomes/emulsions (Section 5.2)
- Ion-exchange resin for removal of endogenous metals (Section 5.3)
- Synthetic antioxidants as positive controls (Section 5.4)
- Zeta-potential in liposomes containing iron (Section 5.5)

5.1 Methemoglobin-mediated lipid oxidation

A series of experiments addressing the prooxidant activity methemoglobin (metHb), including the effect of dietary antioxidants on metHb-mediated oxidation, was performed using the liposomal model system and the oxygen consumption method. For the convenience of the reader, the methodology for these experiments is first presented, followed by the results and a discussion on metHb-mediated oxidation (Sections 5.1.4, 5.1.5, and 5.1.6).

5.1.1 Material

Bovine methemoglobin (metHb) purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) was used for the experiments. An aqueous working solution (1 mg/mL = 15.5 μM) was prepared fresh before each set of experiments, and the solution was kept at an ambient temperature (~22 °C) during the experiments.

Marine phospholipids isolated from washed cod *(Clupea harengus)* roe, obtained from Grøntvedt pelagic (Uthaug, Norway), were used for the preparation of liposomes. Before isolation of the phospholipids, the roe was kept frozen at –40°C. Data on the oxidation status (PV, AV, TBARS), carotenoid content and composition (purity and fatty acid profile) of the isolated phospholipids are available in Table 7.

5.1.2 Methods

Procedures for the isolation of phospholipids, preparation of liposomes and oxygen uptake measurements are described in detail in **PAPER I – IV**. Processing of oxygen uptake recordings is described in Section 4.2. All experiments were performed with 1.5% (w/v) liposomes in 5.5 mM MES solution, pH 5.5. The 1.5% liposome dispersion was prepared by dilution of 3% (w/v) liposome dispersion (30 mg PL/mL) with 5.5 mM MES solution (pH 5.5). Sonication of the 3% liposomes was done with a 6 s pulse repeated 25× (net sonication time = 2.5 min) at an amplitude 50% using a Vibra Cell sonication system with a ∅12 mm sonication rod (Sonics & Materials Inc., Newton, CT, USA) and a test tube (∅2.5 cm). Typically, 15 – 20 mL of liposome dispersion was prepared for a set of experiments. The temperature in the oxygraphic cells was kept at 30°C and the experimental volume of the liposome dispersion was 1 mL in each oxygraphic cell. All experiments were performed in triplicate using three simultaneous oxygraphic cells.

An aliquot of 10 – 80 μL of the metHb work solution was injected into the experimental volume. The concentrations of metHb in the final experimental volume are shown in Table 9. The final experimental volume consisted of a volume of liposome dispersion (1 mL) and the aliquot of added metHb work solution. Concentrations related to the amount of iron contained in methemoglobin (metHb-Fe) were used for expressing methemoglobin concentrations.

For experiments where complete chelation of free metals was desired, 1.71 mM aqueous solution of ethylenediaminetetraacetic acid (EDTA) was injected into the experimental volume, so that 20 – 25-fold mol excess of EDTA in relation to the amount of iron contained in meHb was achieved (shown in Table 9).

For experiments where complete depletion of pre-formed lipid hydroperoxides (LOOH) was desired, triphenylphosphine (TPP) dissolved in chloroform was added to the phospholipids before complete evaporation of chloroform and sonication, so that the concentration of TPP in the prepared liposome dispersion would be 200 μM. The TPP was added in ~50-fold molar excess than the peroxide content determined in the phospholipids (9.7 ± 0.8 mmol LOOH/kg). The excess of TPP used to also deplete any LOOH possibly formed during sonication of phospholipids.

Table 9 Methemoglobin concentrations in the final experimental volume of liposome dispersion, expressed as a molar concentration (μM) of methemoglobin (metHb) and as a molar concentration (μM) and molar quantity (nmol) of iron contained in methemoglobin (metHb-Fe); the last column shows the molar quantity of EDTA (nmol) added to the dispersion for each concentration of metHb where chelation of free metals was desired

Aliquot of metHb work solution (μL)	**metHb** (μM)	**metHb-Fe** (μM)	**metHb-Fe** (nmol)	**EDTA** (nmol)
10	0.15	**0.60**	0.60	12.0
20	0.29	**1.17**	1.20	25.7
40	0.55	**2.21**	2.28	51.3
60	0.78	**3.12**	3.32	77.0
80	0.98	**3.94**	4.24	102.6

Table 10 Combinations of water and lipid soluble antioxidants and methemoglobin in the final experimental volume of liposome dispersion; lipid soluble antioxidants are expressed as a relative quantity (%) in the phospholipids

Antioxidants		Antioxidant concentration (μM or % (g/100 g PL))	Methemoglobin concentration (μM metHb-Fe)
Water soluble (*hydrophilic*)	**Ascorbic acid**	25 μM, 50 μM	2.21
	Caffeic acid	50 μM, 100 μM	2.21
Lipid soluble (*lipophilic*)	**Astaxanthin**	0.1%	2.21, 3.12
		1.0%	2.21, 3.12
	Ascorbyl palmitate	0.1%	2.21, 3.12
		1.0%	2.21, 3.12
	α-Tocopherol	0.1%	2.21
		1.0%	2.21, 3.12
	δ-Tocopherol	0.1%	2.21, 3.12
		1.0%	2.21, 3.12

5.1.3 Addition of antioxidants

A stock solution of ascorbic acid (100 mM) was prepared in 5.5 mM MES solution, a stock solution of caffeic acid (100 mM) was prepared in 96% ethanol, and the solutions were kept at 4°C. Work solutions were prepared fresh before the experiments by diluting appropriate aliquots of the stock solutions with 5 mM MES solution (pH 5.5). An aliquot of the work solution was injected into the cell with the liposome dispersion to achieve the desired concentration in the reaction volume.

Stock solutions of lipid soluble antioxidants, namely astaxanthin (0.2 mM, 2 mM), α-tocopherol (4.6 mM), δ-tocopherol (5.1 mM) and ascorbyl palmitate (4.8 mM), were prepared by dissolving the compounds in chloroform. An aliquot of the stock solution was added to the phospholipids before complete evaporation of chloroform and sonication, to achieve the desired quantity in the liposomes. Levels of 0.1% and 1.0% antioxidant (w/w, phospholipid base) were tested.

The quantities of the antioxidants in the final experimental volume of the liposome dispersion and the concentration of methemoglobin tested with the antioxidants are given in Table 10.

5.1.4 Prooxidant activity of methemoglobin in liposomes

After the addition of metHb to the liposomes, fast consumption of the dissolved oxygen followed, as shown in Figure 19. The rate of oxygen consumption was fastest directly after the addition of metHb, and gradually decreased over time. The net initial OUR, *i.e.* OUR measured shortly after metHb addition, was linearly proportional to the metHb-Fe concentration (Figure 19).

In LMW iron-mediated lipid oxidation the consumption of the dissolved oxygen followed a linear function (**PAPER I**). The consumption of the dissolved oxygen in metHb-mediated oxidation did not follow any of the basic mathematical functions, *i.e.* linear, exponential, quadratic or logarithmic function. For this reason, quantification of the oxygen uptake rates (OUR) became more complicated. Therefore, the results are presented as processed oxygen uptake curves or as the net initial OUR, characterizing the fastest phase in metHb-mediated oxidation. To evaluate the degree of metHb oxidation and the effect of different factors, including antioxidants, the oxygen uptake curves or the net initial OUR were directly compared. The latter approach was used in **PAPER II** and **PAPER IV**.

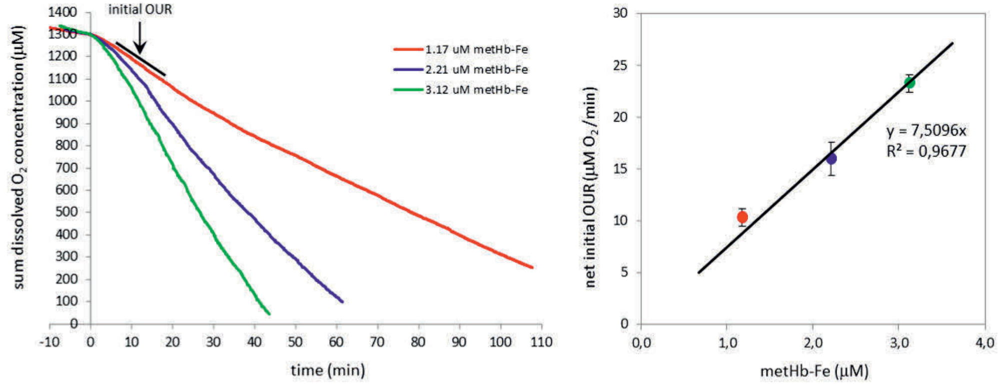

Figure 19 Left: Oxygen uptake in 1.5% liposomes (pH 5.5) after addition of metHb (zero time point); **Right:** Relationship between the net initial OUR and metHb-Fe concentration.

A comparison of the UV-spectrum of the metHb work solution with UV-spectra of different forms of mammalian hemoglobin found in literature [150] confirmed the presence of metHb in the work solution (Figure 20). It has been reported that hydration weakens the heme-protein linkages [151] and that once the heme group of hemoglobin is converted to the met-form (Fe^{3+}) the group is much more loosely bound to the protein. This favours dissociation of the group as hemin (protein-free analogue of metHb) and consequently hemin-mediated lipid oxidation [152]. Further degradation of hemin would result in a release of free iron into the work solution. A product specification sheet for bovine hemoglobin (product no. H2625/Sigma-Aldrich) states that the methemoglobin powder contains 0.25 – 0.30% free iron. Therefore, traces of LMW iron were expected in the metHb work solution.

However, a detailed composition in terms of intact metHb, hemin and free iron of the freshly prepared metHb work solution was not determined. A hypothetical situation, in which all iron would be released from the methemoglobin, would essentially lead to LMW iron-mediated oxidation. The highest metHb concentration (3.94 μM metHb-Fe) used in this study would give ~4 μM LMW iron in the final experimental volume of the liposome dispersion. Fe^{2+}-mediated oxidation in 1.5% liposomes prepared from the same phospholipids as in these experiments was measured in **PAPER I**. Iron concentration of 4 μM Fe^{2+} would give net OUR < 3.1 μM O_2/min, which is a relatively small contribution to the overall OUR.

This also demonstrates that the prooxidant activity of metHb is much higher than the prooxidant activity of LMW iron. While the specific OUR [1-footnote] for Fe^{2+}-mediated oxidation in the 1.5% liposome dispersion was 0.29 ± 0.12 M O_2/M $Fe^{2+}.min^{-1}$ (**PAPER I**), the specific OUR for metHb-mediated oxidation was 7.5 ± 0.9 M O_2/M metHb-Fe.min^{-1} (n = 18) (calculated from the data for the initial net OUR in Figure 19 – Right), which is a 26-fold higher specific OUR than the one for LMW iron, under the same experimental conditions.

[1] specific OUR [M O_2/M $Fe^{2+}.min^{-1}$] = net oxygen uptake rate (OUR) for a given concentration of the prooxidant, divided by the prooxidant concentration

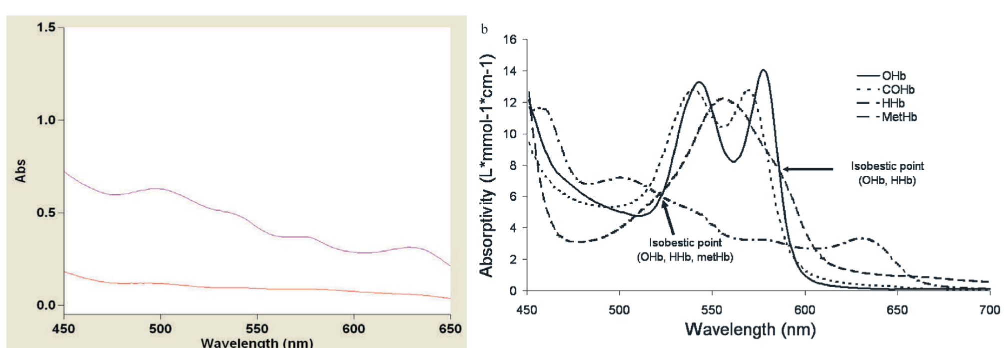

Figure 20 Left: Absorption spectrum (450 – 650 nm) of bovine metHb work solution (15.5 µM metHb) (upper curve) and the dissolution medium (5.5 mM MES solution, pH 5.5) (bottom curve); **Right:** Absorption spectra of different types of mammalian hemoglobin (figure from [150]) used for comparison; metHb is marked ·—·—·—·

A study by Richards *et al.* suggests that dissociation of the heme group has a primary role in the ability of different heme proteins to promote lipid oxidation processes [153]. A comprehensive review by Schaich on heme-mediated lipid oxidation however concludes that an intact heme group inside a pocket formed by surrounding proteins, *i.e.* non-dissociated heme group, was an absolute requirement for the prooxidant activity of heme-proteins [32].

If Richards' [153] hypothesis is correct, then the prooxidant activity of denatured metHb should remain the same or even increase in comparison to the native metHb, because denaturation would aid dissociation of the presumably more active hemin. A lower pro-oxidative activity of denatured metHb would, on the other hand, favour Schaich's [32] hypothesis. Temperatures above 75 °C lead to denaturation of heme-proteins [113].

Therefore, the metHb work solution was boiled (100 °C) in a water bath for 10 min in order to thermally denature the protein and presumably aid dissociation of the heme group. It should be mentioned that some precipitation of protein occurred in the heated metHb solution, which was likely the denatured protein. The effect of the heat treatment on the oxygen uptake consumption in 1.5% liposomes (pH 5.5) is shown in Figure 21. The initial net OUR was reduced by 41 % in the denatured metHb-mediated oxidation, as well as the subsequent OUR. Therefore, thermal treatment reduced the activity of metHb. The results are in agreement with Schaich's hypothesis that the protein part is important for the heme group to rapidly catalyse lipid oxidation.

The activity of metHb was however not reduced completely by the heat treatment. The results suggest that the dissociated heme group still have some prooxidant activity. This activity however appears lower than when the group is embedded in the protein structure.

To see whether LMW iron was released from metHb during the thermal treatment and to what degree this affects the consumption of the dissolved oxygen, a strong chelator (EDTA) was added to the liposomes to deactivate any free iron. The effect of EDTA is shown in Figure 21. A presence of EDTA further reduced the activity of thermally denatured metHb. This suggests that release of free iron from metHb occurred during the heat treatment. These results imply that 1) the prooxidant activity of dissociated heme group (hemin) is lower than when the group is embedded in the protein structure, or 2) the heme-group is largely destroyed by the heat treatment leaving only LMW iron to be active as a prooxidant.

These experiments show that heating of heme-proteins leads to a reduction of their prooxidant activity, and it could therefore be one of the strategies for reduction of heme-mediated lipid oxidation. In further experiments presented in this thesis, the native form of metHb is used.

In a study by Kristensen *et al.* [113], temperatures above the denaturation point of metmyoglobin (metMb) (> 75°C) decreased the pro-oxidative activity of the resulting heat-modified metMb species compared to the native metMb. But even though the pro-oxidative activity of metMb decreased after the heat treatment, the catalytic activity of heat-denatured metMb still exceeded the pro-oxidative activity of free Fe^{2+}. The authors measured a minor loss of free iron from metMb after a short heat treatment but a larger loss during a longer heat treatment. These findings are in a good agreement with the results in this study.

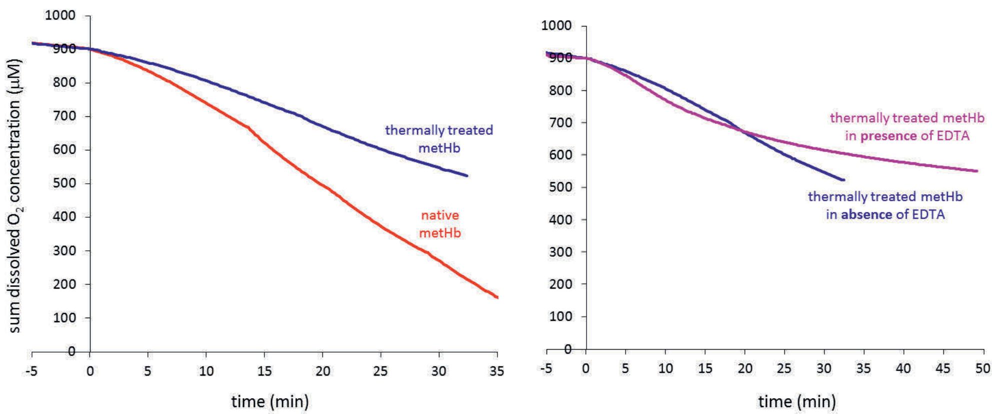

Figure 21 Oxygen consumption curves of lipid oxidation in 1.5% liposomes (pH 5.5) mediated by native metHb (red curve) and thermally treated metHb (10 min in a boiling bath) (blue curve) (**left plot**), and by thermally treaded metHb in the absence (blue curve) and the presence (purple curve) of EDTA (**right plot**). All metHb concentrations in the plots were 2.21 µM metHb-Fe.

5.1.5 Oxygen uptake kinetics in methemoglobin-mediated oxidation

In order to verify the occurrence of LMW iron-mediated oxidation alongside metHb-mediated oxidation and these, EDTA was added into the liposome dispersion to chelate any endogenous trace metals and all the iron which could be released from the native metHb. EDTA was added into the experimental volume either before addition of metHb or during ongoing metHb-mediated oxidation. The oxygen uptake curves are shown in Figure 23.

The presence of EDTA had a dramatic effect on the course of the oxygen consumption. The initial net OUR was reduced by a factor 0.7 (shown in Figure 22) and the rate of oxygen consumption gradually decreased over time, eventually reaching values similar to the background OUR (Figure 23). The change in oxygen uptake kinetics was also clearly seen when EDTA was added into ongoing metHb-mediated oxidation – after the addition of EDTA the subsequent curve resembled the curve for EDTA added prior to addition of metHb (shown in Figure 23). The specific OUR for metHb-mediated oxidation in the presence of EDTA was 4.7 ± 0.9 M O_2/M metHb-Fe.min^{-1} (n = 30) (calculated from the data for the initial net OUR in Figure 21 – Left plot). The reduction in the specific OUR caused by the presence of EDTA is therefore 2.8 M O_2/M metHb-Fe.min^{-1}. This difference could be partially attributed to LMW iron potentially released from metHb and endogenous metals in the liposomes dispersion. However, the difference cannot be entirely attributed to free metal-mediated oxidation (both endogenous metals and LMW iron potentially released from metHb) because the specific OUR for LMW iron-mediated lipid oxidation was found to be 0.29 ± 0.12 M O_2/M Fe^{2+}.min^{-1} (**PAPER I**). A possible explanation for the difference could be that EDTA has an inhibition effect on the activity of metHb, reducing the activity by 33%. These experiments also indicate that LMW metal-mediated oxidation might occur alongside metHb-mediated oxidation.

The effect of EDTA on the activity of hemoglobin (Hb) from Atlantic pollock was investigated by Maestre *et al.* [154] The authors did not find any significant influence of EDTA (up to 1 mM) on pollock Hb (3 μM)-mediated lipid oxidation in a liposomal model (0.8% soy lecithin, pH 6.8, 30°C), measured as formation of TBARS and conjugated dienes (CD) over a 4.5 h period. This finding is in contrast to findings in this study. On the other hand, the authors measured an inhibition of TBARS, CD and a reduced loss of redness over a 4 day period at 4°C in washed fish muscle containing 3 μM of pollock Hb/kg and 2 mmol EDTA/kg muscle, compared to a control lacking EDTA. These findings, on the other hand, support the outcomes of this work. The authors concluded that iron chelators showed lower antioxidant activity on Hb-mediated lipid oxidation than reductants, such as ascorbic acid and proanthocyanidins.

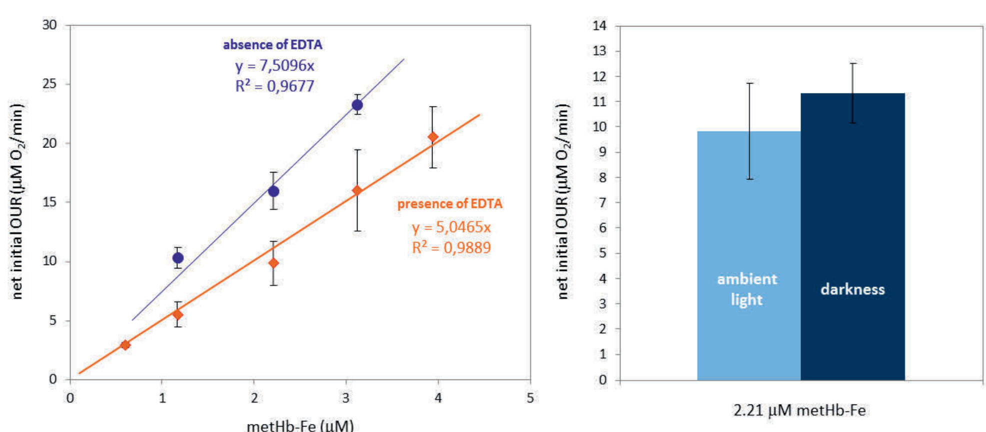

Figure 22 Left plot: Relationship between metHb concentration and net initial OUR when EDTA was absent or present in the liposome dispersion; **Right plot:** Net initial OUR in oxidation mediated by 2.21 µM metHb-Fe at ambient light or in the darkness. The data are average values with respective standard deviations of 3 – 6 measurements in 1.5% liposome dispersion at pH 5.5.

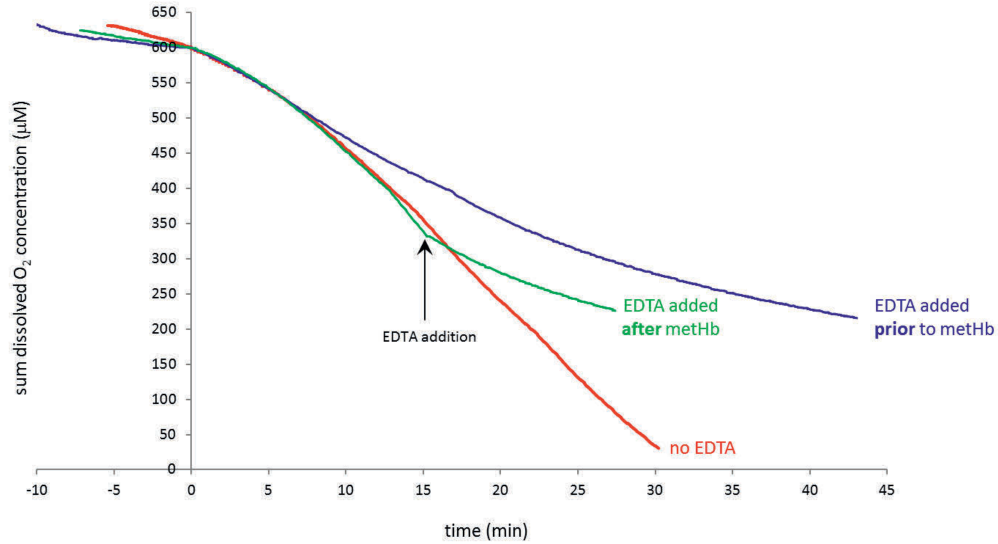

Figure 23 Oxygen uptake curves in 1.5% liposomes (pH 5.5) after addition of 2.21 µM metHb-Fe (zero time point) when EDTA was absent (red curve), added prior to addition of metHb (blue curve), or added after metHb, *i.e.* during ongoing metHb-mediated oxidation (indicated by arrow).

The effect of EDTA on metHb-mediated oxidation needs to be deeper investigated due to the inconsistencies in the reported data. Since it was of interest in this thesis to study metHb-mediated

oxidation only, EDTA was always added into the experimental volume of the liposome dispersion prior to addition of metHb in quantities given in Table 9 to primarily supress (filter out) oxidation mediated by LMW iron and by endogenous metals. The reducing effect of EDTA (by 33%) on the activity of metHb was considered throughout the study. Unless specified otherwise, all the following experiments were performed with liposomes dispersions containing EDTA.

Heme-proteins are known to have photosensitising abilities, *i.e.* they are capable of converting energy of light into chemical energy (more details on the mechanism is given in Section 3.2.2.1). Type II photosensitizers convert atmospheric triplet oxygen ($^3O_2$) into reactive singlet oxygen ($^1O_2$) which is capable of direct reaction with double bonds in unsaturated fatty acids, forming lipid hydroperoxides (LOOH). Assuming that the highly reactive singlet oxygen is generated by the heme-group in methemoglobin and that the generation is responsible for oxygen consumption, metHb-mediated oxidation should have higher oxygen uptake rates in the light compared to in the dark, and peroxidation may even not occur in the dark. Oxygen uptake curves for 2.21 μM metHb-Fe measured in complete darkness were not significantly different from the curves measured at ambient daylight (expressed as initial net OUR in Figure 22 right plot). These results suggest that generation of singlet oxygen was not a dominant pathway in metHb-mediated oxidation in the liposome system.

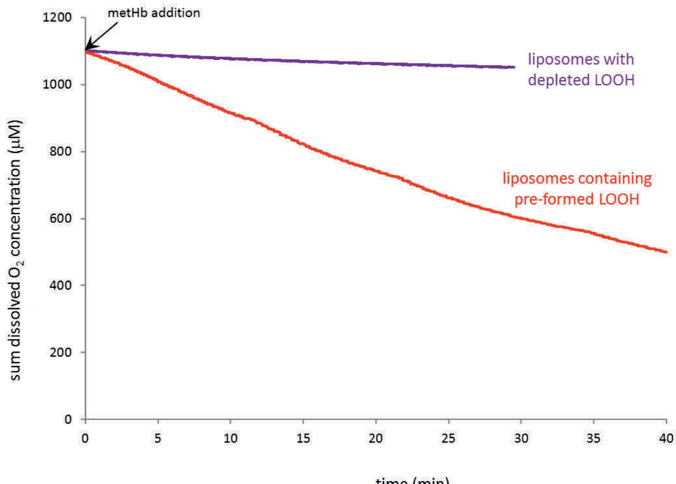

Figure 24 Oxygen consumption curves in 1.5% liposomes (pH 5.5, incl. EDTA) after addition of 3.94 μM metHb-Fe when the pre-formed lipid hydroperoxide (LOOH) content in the phospholipids was kept (red curve) or depleted by reaction with TPP (200 μM) which was added to the phospholipids prior to preparation of liposomes.

Pre-formed lipid hydroperoxides (LOOH) have been proposed to play a key role for heme-proteins to be active in mediating lipid oxidation [32]. It is possible to chemically reduce the hydroperoxide group (–OOH) into an alcohol group (–OH) by triphenylphosphine (TPP). To verify the interactions of metHb with LOOH, TPP was added to the phospholipids in order to prepare LOOH-free liposomes. The oxygen uptake in these liposomes after addition of 3.94 uM metHb-Fe is shown in Figure 24. The oxygen uptake was completely supressed by elimination of the pre-formed LOOH, which confirms that the presence of LOOH is essential for metHb to be prooxidatively active.

When oxygen consumption after addition of metHb was followed for a longer period (1 – 3 h), the OUR eventually reached OUR similar to the OUR measured before the addition of metHb, *i.e.* the background OUR. Assuming that the background oxygen uptake rate is constant, it is possible to subtract the background oxygen consumption from the consumption measured for metHb, obtaining a *net oxygen uptake curve* for metHb. When approximating this net curve to zero along the x-axis, the curve was well fitted with a basic exponential function ($y = Ae^x$) (shown in Figure 25).

The net oxygen consumption curve clearly shows that the oxygen uptake in metHb-mediated oxidation decreases exponentially, and after some time approaches zero. This result suggests that metHb loses its initial prooxidant activity while mediating lipid oxidation. At this moment, the cause for the decrease in the prooxidant activity of metHb is not clear.

Grunwald and Richards [155], studying myoglobin (Mb)-mediated lipid oxidation in washed cod muscle, also observed a decreased activity of certain types of heme-proteins after dissolution in aqueous medium. The authors proposed a mechanism in which the pro-oxidation activity of Mb is reduced due to destruction of the hemin group by interaction with pre-formed lipid hydroperoxides (LOOH). A few years later, Meastre *et al.* [156] reported that different lipid oxidation by-products (hydroperoxides and aldehydes) accelerate both hemoglobin oxidation to metHb and hemin release and that fish hemoglobins with stronger prooxidant capacities are those with weaker resistance to undergo oxidation to metHb and hemin loss. These findings could also be interpreted as followed: Intact hemoglobin is a stronger prooxidant than when it is converted to metHb and further degraded. The proposed mechanism is summarised in Figure 26. This theory would explain the exponential decrease of oxygen uptake in metHb-mediated oxidation in this work.

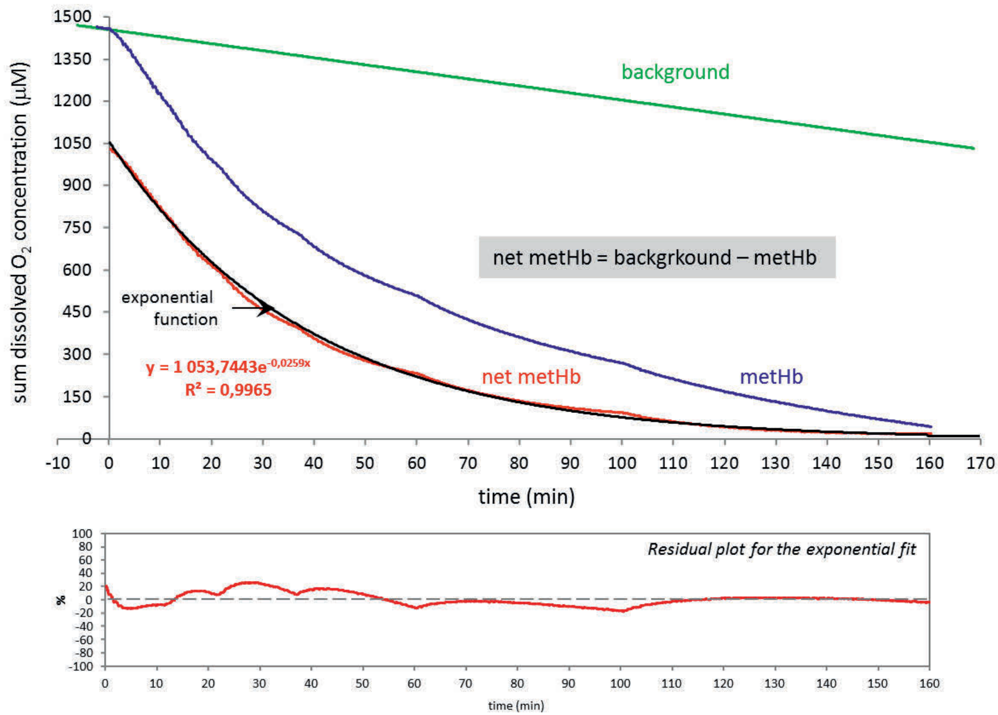

Figure 25 Upper plot: The principle of obtaining a *net oxygen uptake curve* (red curve) for metHb-mediated oxidation (3.94 μM metHb-Fe) by subtraction of the background linear oxygen uptake curve (green curve) from the measured curve for metHb (blue curve). A basic exponential function is fitted the net curve. **Lower plot:** Residual plot for the exponential fit expressed as % deviation of the curve from the exponential function.

Roginsky *et al.* [111] also observed a decrease in the prooxidant activity of horse heart metMb with time during oxidation of methyl linoleate in Triton X-100 micellar solution at 37°C. This effect was shown to be caused by degradation of iron-heme complexes. The authors also showed that degradation of metMb occurs in the presence of oxidizing lipids and that molecular oxygen does not participate in the process of metHb/heme degradation but lipid hydroperoxides are involved in the process. In addition, the authors determined some kinetic parameters characterizing the catalytic activity of hemin and metMb in the experimental system. An oxygen uptake method, similar to that in this thesis, was used in their studies, and their findings are in very good agreement with the measurements in this study.

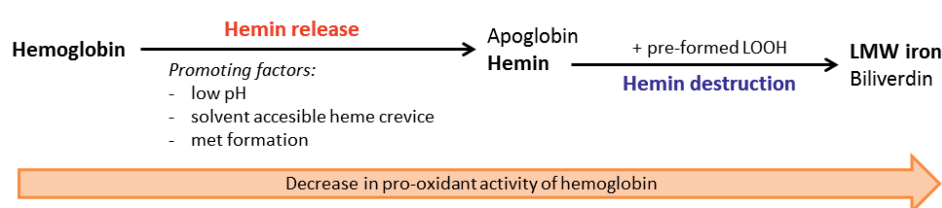

Figure 26 Proposed reaction pathway for decrease in the prooxidant activity of methemoglobin (adapted from [155])

Concentrations ranging 0.60 – 3.94 μM metHb-Fe were added to the liposomes dispersion containing EDTA and the oxygen consumption was recorded until the OUR reached background values. The net oxygen uptake curves were generated in the same way as the example in Figure 25, representative net curves for the concentration range are shown in Figure 27. An exponential decrease in oxygen consumption was observed for all measured concentrations.

This consistency in the curves and the fact that it was possible to repeatedly re-saturate the liposome dispersion with air in order to obtain longer oxygen uptake recordings suggest that neither the dissolved oxygen concentration, nor the level of pre-formed LOOH, nor the amount of double bonds in the fatty acids were limiting factors for metHb-mediated oxidation, given that the latter two variables were in excess to metHb concentration for the duration of the experiment.

The exponential fits provided data on the total consumption of the dissolved oxygen and the rate constant (k) of the oxygen consumption. Their relationship to metHb concentration is plotted in Figure 28. The total consumption of the dissolved oxygen was linearly proportional to the metHb concentration. The rate constant for the consumption appeared constant (k = 0.030 ± 0.005 min^{-1}, n = 17) over the measured concentration range of metHb.

The ability of metHb to rapidly mediate lipid oxidation can be clearly seen from the total amount of consumed oxygen per metHb molecule. Each mol of iron contained in the methemoglobin molecule (metHb-Fe) was responsible for the consumption of 242 ± 47 mol of dissolved O_2 in the liposome dispersion (calculated from data in Figure 28 Left). In contrast, each mol of LMW iron was responsible for the consumption of 5.94 ± 0.26 mol O_2 in the same system and under the same conditions (**PAPER I**), which is a 40-fold lower value. This again, demonstrates a much higher prooxidant activity of metHb compared to LMW iron. However, metHb is not a true catalyst, like LMW iron, since its activity decreases over time, as shown and discussed above.

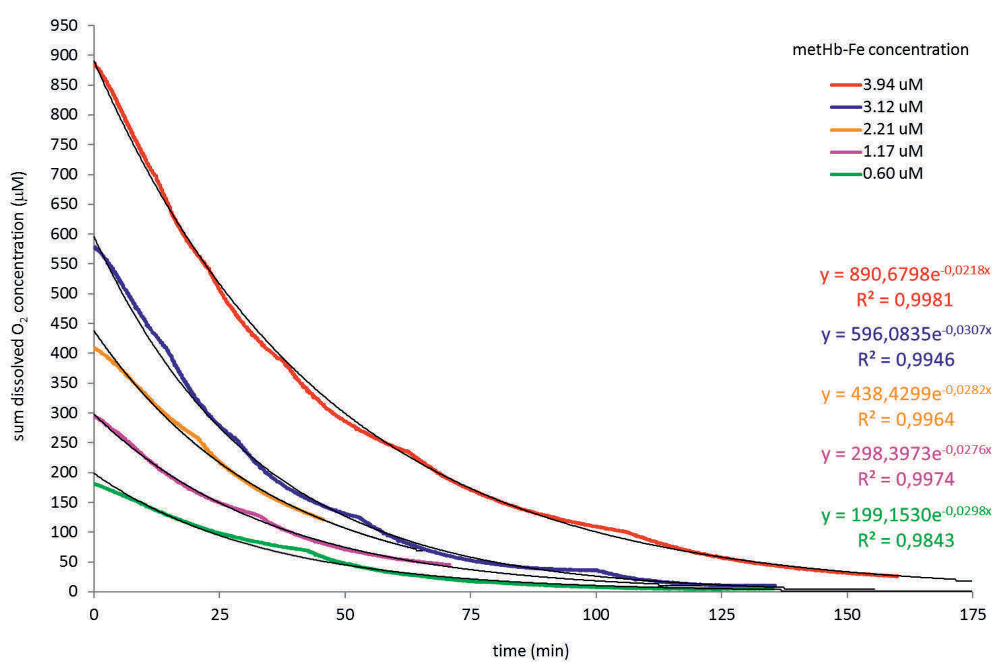

Figure 27 Net oxygen uptake curves for metHb-mediated lipid oxidation in 1.5% liposome dispersion (pH 5.5, incl. EDTA) in the range 0.60 – 3.94 µM metHb-Fe. The curves are fitted with basic exponential functions.

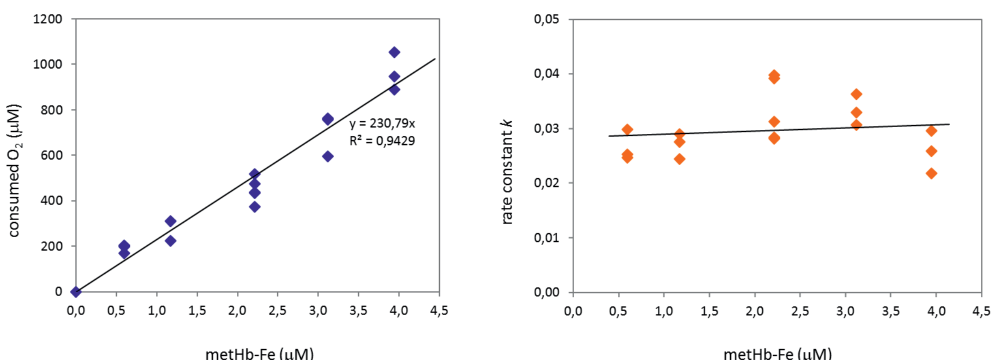

Figure 28 The relationship between metHb-Fe concentration and the total consumption of the dissolved oxygen (**left plot**) and the rate constant (**right plot**), both determined by the exponential fits through net oxygen uptake curves for metHb-mediated oxidation in 1.5% liposomes (pH 5.5, incl. EDTA). Each data point in the plots represents one measurement.

5.1.6 Effects of antioxidants

The activity of antioxidants is affected by interactions with LMW iron, which may turn some of the antioxidants into prooxidants, as shown in **PAPER III**. Caffeic acid was strongly pro-oxidative in the presence of LMW iron, but inhibited metHb-mediated lipid oxidation in the liposome dispersion (pH 5.5), which demonstrates that the activity of some antioxidants is also dependent on the type of prooxidant (shown in **PAPER II**). Therefore, the antioxidant activity of important dietary antioxidants (ascorbic acid, caffeic acid, astaxanthin, α- and δ-tocopherol, and ascorbyl palmitate) was, in addition to LMW iron-mediated oxidation, also evaluated in metHb-mediated oxidation. To eliminate the interference of LMW iron (both endogenous and released from metHb), EDTA was added into the liposome dispersion in all experiments (according to Table 9).

Representative oxygen consumption curves for metHb-mediated oxidation (2.21 and 3.12 μM metHb-Fe) in 1.5% liposome dispersion (pH 5.5) containing EDTA and the antioxidants are shown in Figure 29 and Figure 30. Direct comparisons of the oxygen uptake curves clearly show that none of the antioxidants at the tested concentrations and ratios to metHb had a pro-oxidative effect. In LMW-iron mediated oxidation (**PAPER III**), caffeic acid and α-tocopherol showed pro-oxidative behaviour due to the ability to reduce LMW ferric iron (Fe^{3+}) to the more prooxidative ferrous state (Fe^{2+}) and ascorbic acid was depleted by reactions with LMW iron. Iron in metHb is "complexed" by the porphyrin and protein structure. The absence of the prooxidant effect for these antioxidants shows that whether the iron atom is free or embedded in the heme group is crucial for the direct interactions between the iron and the antioxidants. An impaired access of the antioxidants to the iron in the heme-group, especially the lipophilic antioxidants, could play a role for the interactions between the iron in heme and antioxidants.

The oxygen uptake kinetics was significantly affected in the presence of the antioxidants, especially at the higher concentrations of the antioxidants (1%, w/w, PL base). While for the control (no antioxidant), the oxygen consumption as a function of time followed an exponential curve, in the presence of antioxidants the oxygen consumption often was constant.

The relative effect (%) on the inhibition of the net initial OUR for oxidation mediated by 2.21 μM metHb-Fe and 3.12 μM metHb-Fe is shown in Figure 31A and Figure 31B, respectively. Ascorbic acid had a stronger inhibition effect than caffeic acid (compared at 50 μM). The relative efficiency of the lipophilic antioxidants had the following order:

At 0.1%: α-Tocopherol > δ-Tocopherol ≈ Astaxanthin ≈ Ascorbyl palmitate

At 1.0%: Ascorbyl palmitate > δ-Tocopherol > α-Tocopherol > Astaxanthin

These results clearly show that the effectiveness of these antioxidants against metHb-mediated oxidation is concentration dependent and may switch from inactivity at low concentration to a very high activity at higher concentrations (as seen for ascorbyl palmitate).

The antioxidants may inhibit metHb-mediated oxidation by 1) direct interaction with metHb (presumably reducing hyper active ferryl-hemoglobin species back to metHb) and/or by b) scavenging of radicals generated during the mediated oxidation. Interactions of the antioxidants with LMW iron are not likely, since any LMW metals were deactivated by addition of EDTA into the liposome dispersion.

Ascorbic acid and caffeic acid are water soluble antioxidants, but also reductants, as shown in **PAPER II** and **PAPER III**) and reported in the literature [67, 157]. Both compounds had an inhibition effect on metHb-mediated oxidation; the effect was higher for ascorbic acid than for caffeic acid (compared at 50 μM). Possible inhibition mechanisms of metHb-mediated oxidation by caffeic acid are discussed in **PAPER II**. Briefly, the literature survey in the paper suggests that reduction of the hyper active ferryl-hemoglobin species leads to inhibition of meHb-mediated oxidation.

Inhibition of the formation of peroxide value, TBARS and volatiles by caffeic acid (200 ppm) in washed mince from bighead carp (pH 6) with added hemoglobin was measured by Thiansilakul *et al.* [158]. In a study of Maestre *et al.* ascorbic acid reduced to some extent formation of lipid hydroperoxides, but was not able to diminish generation of TBARS in liposomes during pollock Hb-mediated oxidation [154]. A significant correlation between the electron-donating capacity of different phenolic compounds (amongst them caffeic acid) and their ability to inhibit lipid oxidation in minced fish muscle containing catalytic amounts of hemoglobin was found in a study of Medina *et al.* [159]

In these studies and the present thesis, compounds with reducing abilities demonstrated a positive inhibition of hemoglobin-promoted lipid oxidation. These findings suggest that the reduction of ferrylHb species could be the antioxidant mechanism involved. To what degree these compounds are involved in radical scavenging in the phospholipid bilayer is not known. Given their primary location in the aqueous phase, the contribution to the scavenging of LOOH could be marginal.

The location of the lipophilic antioxidants (α- and δ-tocopherol, astaxanthin and ascorbyl palmitate) is bound to the lipid phase, *i.e.* in the case of liposomes to the phospholipid bilayer due to their non-polar character. These compounds are primarily radical scavengers. Therefore, their efficiency observed in this study could be attributed mainly to scavenging lipid radicals generated during metHb-mediated peroxidation, rather than to direct interactions with metHb. The antioxidant mechanisms of these compounds are introduced in Sections 3.3.2.2, 3.3.3.1 and 3.3.2.3, therefore they will not be repeated here.

5.1.7 Summary of metHb-mediated oxidation

In conclusion, the prooxidant activity of metHb in the liposome dispersion was confirmed to be crucially dependent on the presence of pre-formed lipid hydroperoxides within the fatty acids of the phospholipids and on the complete structure of the methemoglobin molecule. The activity of metHb was not affected by the lack of light. Heat treatment (thermal denaturation) reduced the activity of metHb as well as the presence of EDTA. The results imply that a partial release of iron occurs during thermal denaturation. The prooxidant activity of metHb was confirmed to be much higher than the prooxidant activity of LMW iron.

The oxygen consumption in metHb-mediated oxidation followed a basic exponential function, and the consumption was not limited by the concentration of the dissolved oxygen, as long as the oxygen was present (as seen by the assembly of the curves after the oxygen uptake recordings). MetHb-mediated oxidation was also not limited by the quantity of the pre-formed LOOH and the double bonds in the fatty acid carbon chains, given their excess in the phospholipids in relation to the concentration of metHb.

The exponentially decreasing oxygen consumption demonstrates that the prooxidant activity of metHb is not constant, as in the case of LMW iron. This suggests that meHb has the ability to rapidly promote lipid oxidation, but it is not a true catalyst, as LMW iron. Based on these measurements it is hypothesized that the metHb molecule is degraded when mediating lipid oxidation, which seems to lead to formation of degradation products of metHb with lower prooxidant activity than the activity of intact metHb.

All tested antioxidants (caffeic acid, ascorbic acid, ascorbyl palmitate, astaxanthin, α-tocopherol, and δ-tocopherol) showed an inhibition effect on metHb-mediated oxidation. The extent of inhibition was dependent on the concentration of the antioxidant and metHb-to-antioxidant ratio. Based on the results in this study, the antioxidant strategy for inhibition of metHb-mediated oxidation in multiphase systems could be as follows: a metal chelator should be present to deactivate any endogenous metals and iron released from metHb. A water soluble compound with reducing abilities should be present to attenuate the prooxidant activity of metHb before methemoglobin is destroyed by promoting lipid oxidation, while lipophilic radical scavengers should be present in the lipid phase to inhibit peroxidation caused by metHb during the active period of metHb.

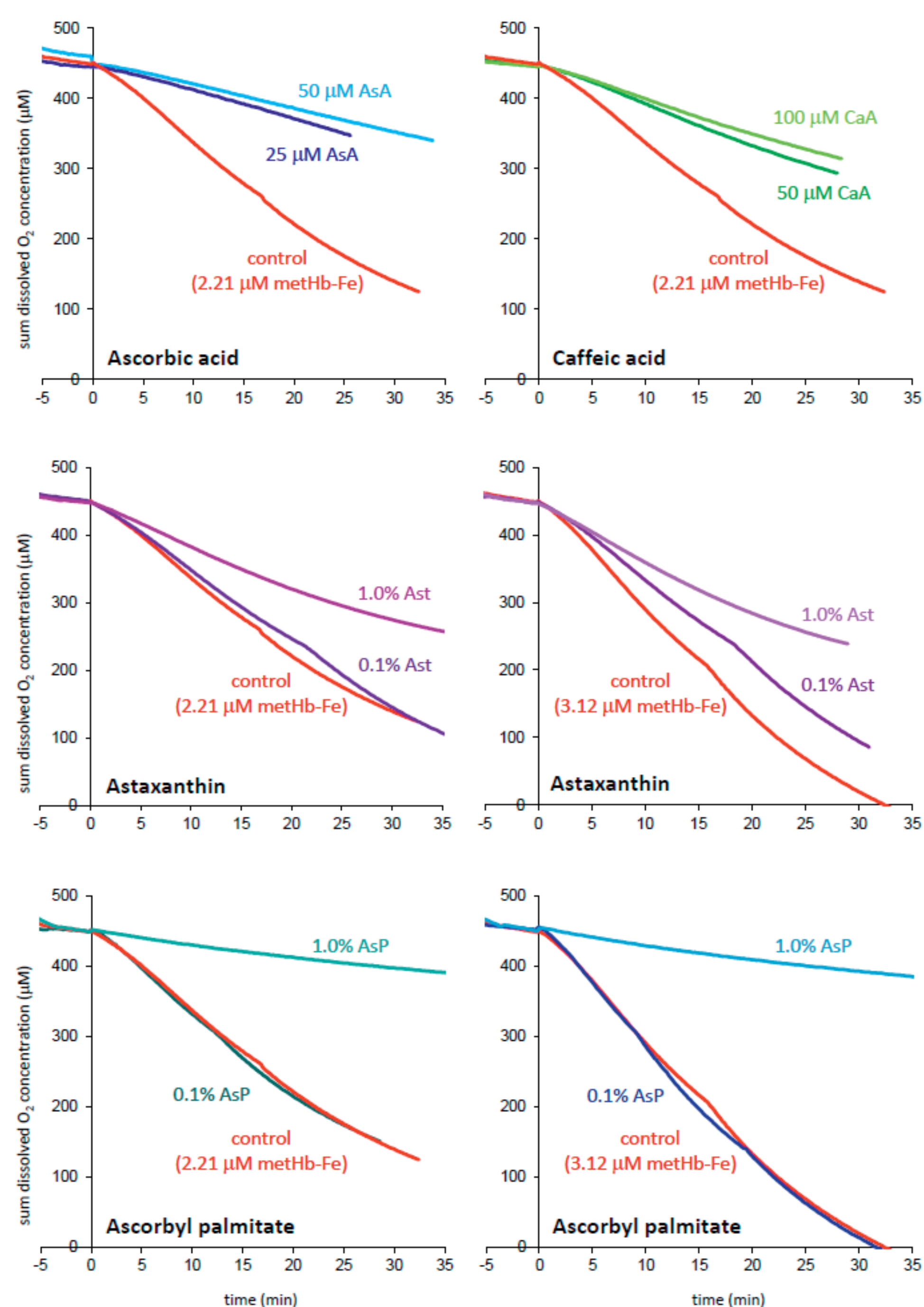

Figure 29 Representative oxygen consumption curves in 1.5% liposome dispersion (pH 5.5, incl. EDTA) containing ascorbic acid (AsA, 25 and 50 μM in the liposomes dispersion), caffeic acid (CaA, 100 and 50 μM in the liposome dispersion), astaxanthin (Ast, 0.1 and 1.0% (w/w, PL base) or ascorbyl palmitate (AsP, 0.1 and 1.0% (w/w, PL base) for metHb-mediated oxidation at 2.21 μM or 3.12 μM metHb-Fe.

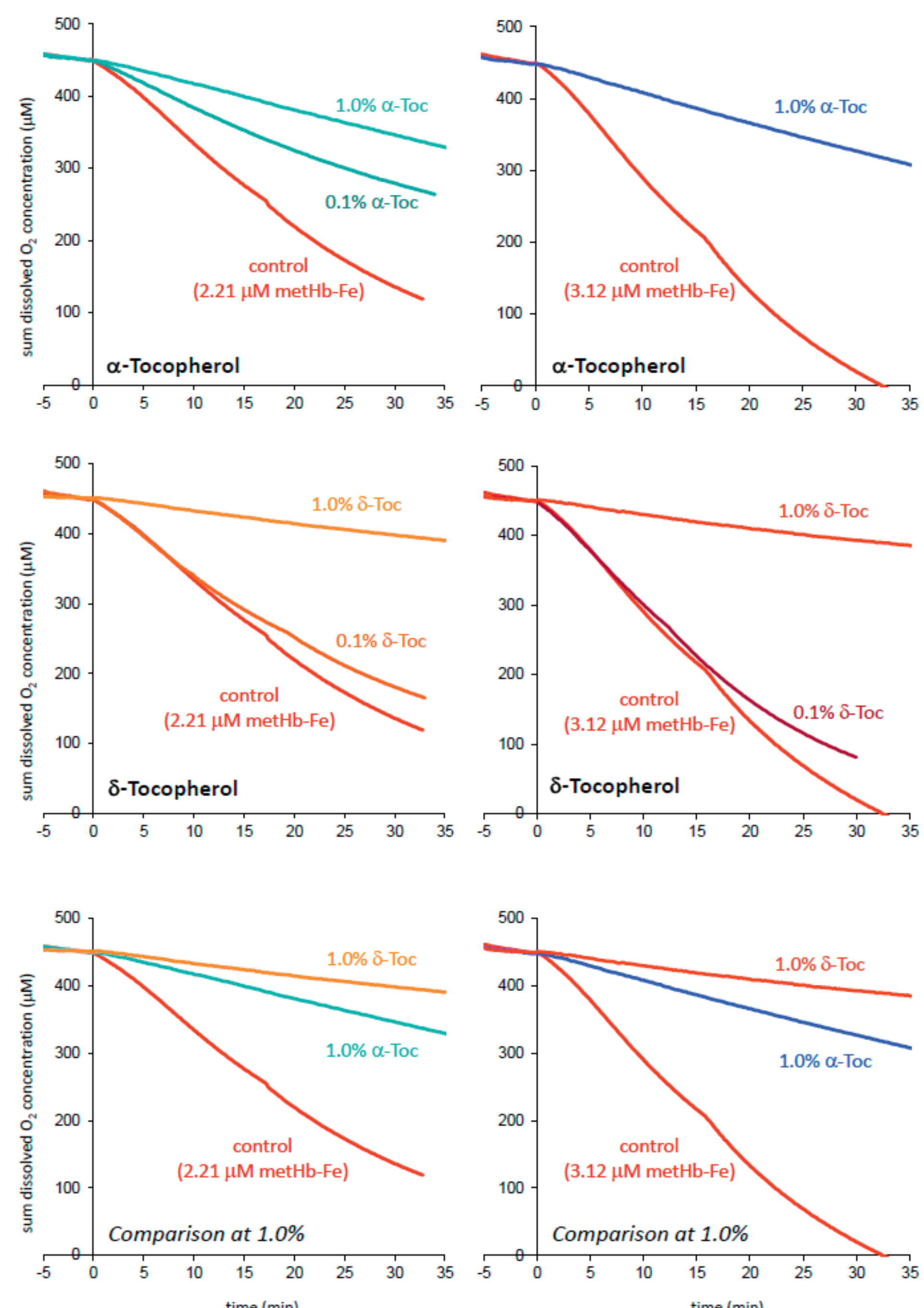

Figure 30 Representative oxygen consumption curves in 1.5% liposome dispersion (pH 5.5, incl. EDTA) containing α-tocopherol (α-Toc, 0.1 and 1% (w/w, PL base) or δ-tocopherol (δ-Toc, 0.1 and 1.0% (w/w, PL base) for metHb-mediated oxidation at 2.21 µM or 3.12 µM metHb-Fe. The curve for 0.1% α-Toc at 3.12 µM metHb-Fe was not available.

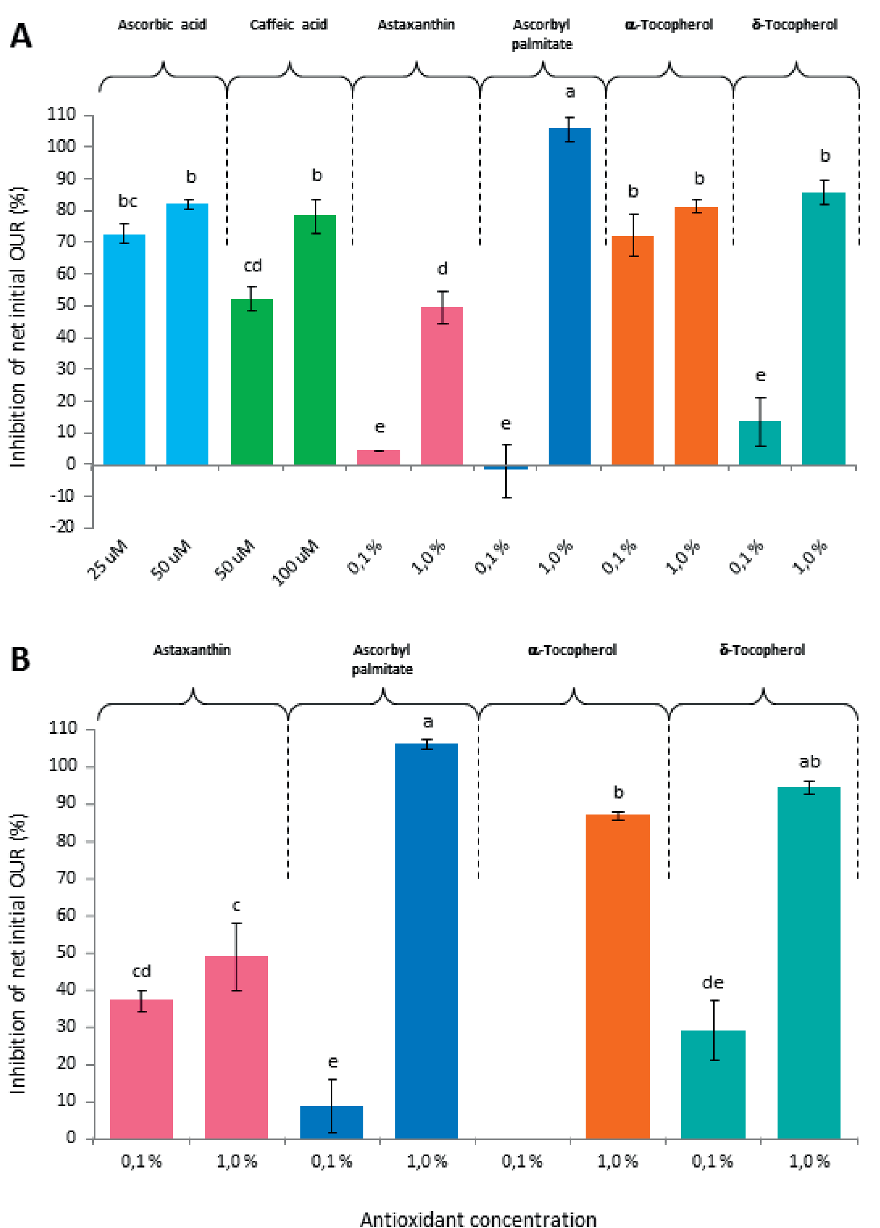

Figure 31 Inhibition (%) of the net initial OUR in oxidation mediated by 2.21 µM metHb-Fe (**plot A**) and 3.12 µM metHb-Fe (**plot B**) by different antioxidants. The concentration of hydrophilic antioxidants (ascorbic acid, caffeic acids) is given in µmol/dm^3 (µM) of the liposome dispersion, the concentration of lipophilic antioxidants (astaxanthin, ascorbyl palmitate, α- and δ-tocopherol) is given as a relative quantity of the antioxidant in the phospholipids (%, w/w, PL base). The data are average values with respective standard deviations of 3 – 6 measurements. Means that do not share the same letter are significantly different (p = 0.05). Data for 0.1% α-tocopherol at 3.12 µM metHb-Fe were not available.

5.2 Endogenous metals in liposomes/emulsions

Contamination by free metals (presumably iron) was expected in the isolated phospholipids and consequently in the liposomes and emulsions stabilized with the phospholipids. Several sources of iron contamination are possible:

1) steel equipment used during isolation of phospholipids,
2) traces of metals in chemicals,
3) metals in the raw material (roe),
4) where sonication was involved (liposomes), the steel sonication rod, and
5) where homogenization was involved (emulsions), the steel homogenization blade.

Phospholipid heads are zwitterions, *i.e.* they bear both positive and negative charge [35]. It is therefore likely that positively charged metal atoms associate with the phospholipid molecules, and due to this they occur in the liposome dispersions and emulsions. Two types of experiments were performed to verify the presence of endogenous metals in the liposomes: the effect of ethylenediaminetetraacetic acid (EDTA) and the effect of ascorbic acid on background oxygen uptake rate (OUR) were tested.

EDTA is a strong synthetic metal chelator. Endogenous metal-mediated oxidation should therefore be inhibited due to complexation of the metals which would be measurable as inhibition of the background oxygen uptake rate. Ascorbic acid, on the other hand, is a strong reducing agent capable of reducing ferric iron (Fe^{3+}) to more active ferrous iron (Fe^{2+}). Ascorbic acid may also act as an oxygen scavenger. Removal of oxygen from the system is facilitated by interactions with reduced metals [160] (**PAPER III**). Both cases would be measurable as increase in background OUR.

The effect of EDTA (17.1 μM) and ascorbic acid (50 μM) (concentrations in the liposome dispersion) on the background OUR in liposomes made from cod roe phospholipids (PL) at pH 5.5 is shown in Figure 32. EDTA significantly reduced (−66%) the background OUR while ascorbic acid significantly increased (+51%) the background OUR. Based on these results, it is reasonable to assume that endogenous metals were present in the liposome dispersion. The effect of EDTA and ascorbic acid on the background OUR is also presented in **PAPER III**, in which emulsions stabilized with herring PL were used.

Background OUR close to the base values, *i.e.* OUR in MES solution which occurs due to consumption of O_2 by the electrode itself [115], would be expected after complete deactivation of iron. Such OUR was however not measured. Peroxidation of fatty acids not catalysed by iron (autoxidation) is expected to run alongside metal-mediated oxidation and/or a proportion of the endogenous iron might not be available for EDTA. Particularly, the iron ions associated with the

phospholipid heads facing the inner cavity of the liposome vesicles, which is separated by the double layer of phospholipids from the outer aqueous environment, could still catalyse oxidation of fatty acid despite addition of sufficient amounts of EDTA.

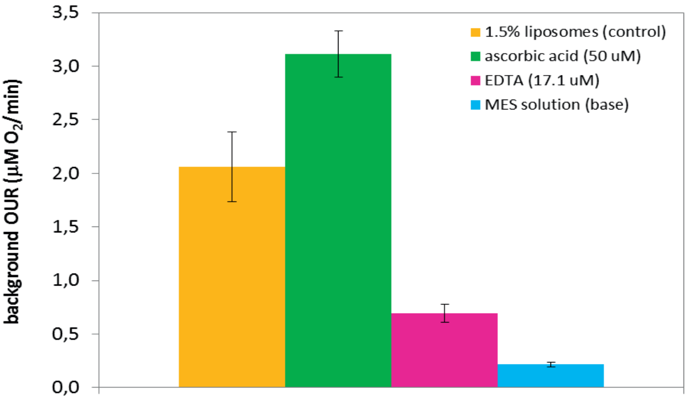

Figure 32 The effect of ethylenediaminetetraacetic acid (EDTA) (17.1 μM) and ascorbic acid (50 μM) on the background oxygen uptake rate (OUR) in 1.5% liposomes (pH 5.5) prepared from cod roe phospholipids. The results are given as the mean values ± standard deviation of 3 – 12 parallels.

Instruments for accurate determination of trace atomic metals in aqueous solutions were not available in the laboratories of SINTEF/NTNU. However, approximation by means of the oxygen uptake rate measurements gave at least a tentative quantification of the endogenous metals. The principle behind the approximation method is illustrated in Figure 33. OUR for different concentrations of added iron were measured and the relationship was plotted. The intersection of the linear trend line of this relation and the base line, *i.e.* OUR in the MES solution (typically 0.15 – 0.35 μM O_2/min), was found, and the value represented an estimate of an endogenous iron level. The highest value determined by this method was found in raw herring roe phospholipids (17.3 mg/kg PL (ppm)).

For the interpretation of data from the oxygraphic experiments the interference of the endogenous iron had to be considered. Added components, such as some antioxidants, might interact with the endogenous iron (as demonstrated on ascorbic acid) giving room for erroneous interpretation of results.

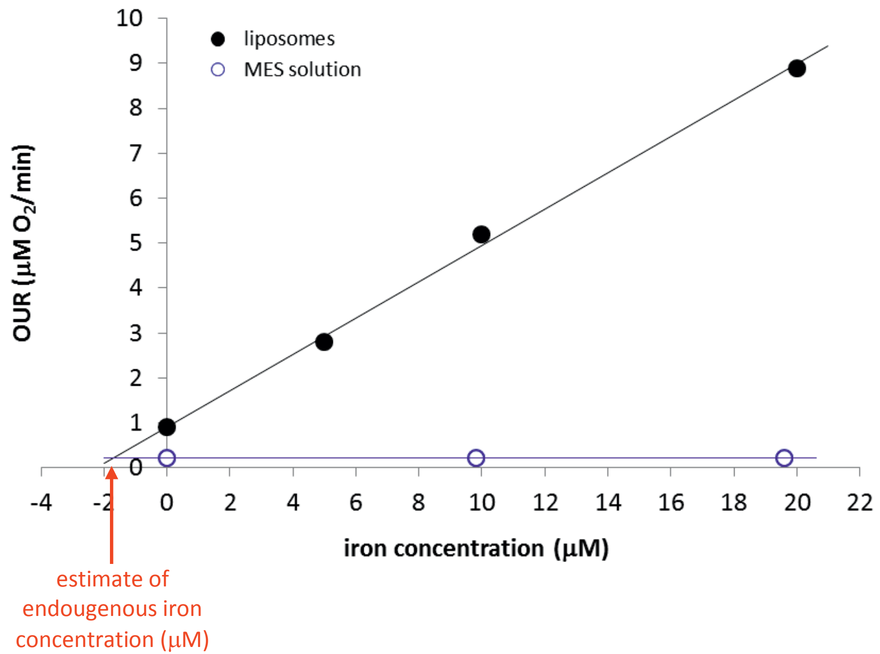

Figure 33 Illustration of estimating the endogenous metal concentration in liposomes by an approximation method, using Fe^{3+} as a standard, which was used for estimate of endogenous iron content in phospholipids

5.3 Ion-exchange resin for removal of endogenous metals

It was desirable to remove the endogenous iron/metals present in the liposome dispersions, because they could interact with added compounds and complicate the interpretation of results. Ion-exchange resins can be used for purification of various liquids from contaminant/poisonous metals. Ion-exchange resins are insoluble organic polymers, typically in the form of beads (∅0.5 – 1 mm) which are highly porous providing a high surface area. The trapping of metal ions occurs with concomitant release of other ions (typically Na^{+}, Ca^{2+}, H^{+}) [161].

Beads of ion-exchange resin (Dowex 50×8, H-form, 20-50 mesh purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) were mixed with a 3% liposome dispersion (pH 5.5) in a concentration of 62.5 mg resin/mL of liposome dispersion. The mixture was incubated for 60 min at 4°C with occasional stirring, followed by removal of the beads from the liposome dispersion by filtration through glass wool. The background oxygen uptake rate in the resin treated liposome dispersion was compared with the values for non-treated liposome dispersion (Figure 34). The background OUR was significantly reduced (by 33%) in the resin treated liposomes and the activity of added ascorbic acid was reduced (by 33%), indicating that 33% of iron was removed. Higher quantities or different types of resin and optimization of the incubation conditions could lead to

better results, and, in the best scenario, even meet the performance of EDTA. On the other hand, if iron is firmly associated with phospholipid heads (as postulated in **PAPER I**), ion-exchange resin might not be capable of efficiently retrieving the iron ions from the phospholipid heads and a complete removal of iron would not be feasible.

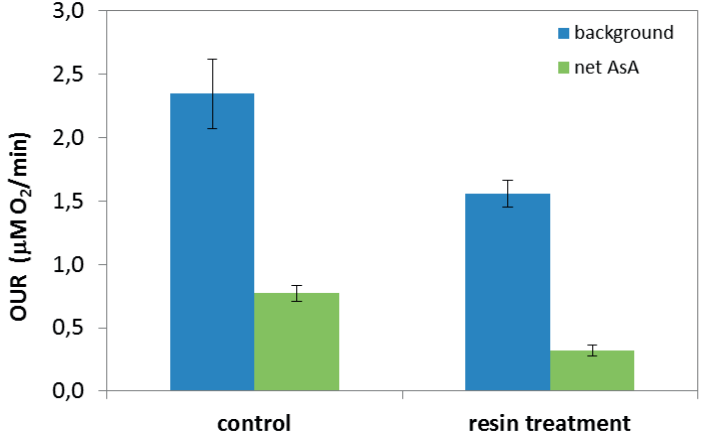

Figure 34 Effect of resin treatment on the background OUR and the net OUR for added ascorbic acid (AsA) (50 µM) and added Fe^{2+} (10 µM) in 3% liposome dispersion (pH 5.5) from cod roe phospholipids. For the OUR measurements, the dispersion was diluted to 1.5% with the MES solution, pH 5.5. The results are given as the mean values ± standard deviation of 3 parallels.

A major drawback in using Dowex H-form resin was acidification of the liposome solution by released (exchanged) H^+ from the resin. Quantities of the resin exceeding 200 mg/mL of liposome dispersion lowered the pH in the liposome dispersion by more than 1 pH unit. Thus, despite partial removal of metals from the liposome solution by the ion-exchange resin at 62.5 mg resin/mL, which was accompanied with a marginal degree of acidification, it was decided not to include the resin treatment into liposome preparation.

5.4 Synthetic antioxidants as positive controls

Synthetic antioxidants, such as butylated hydroxytoluen (BHT) and hydroxyanisol (BHA), *tert*-butylhydroquinone (TBHQ), propyl and octyl gallate (PG, OG) and ethoxyquin (EQ), were designed for high efficiency in inhibition of lipid oxidation in different lipid systems and at variable conditions. These synthetic compounds provide excellent reference values (positive control) to natural antioxidants.

Propyl gallate was used as a positive control In **PAPER II** and **PAPER III** since it was found efficient in inhibiting both metHb- and LMW iron-mediated oxidation. In addition, in Fe-mediated oxidation propyl gallate did not affect the linearity of oxygen uptake curves after addition of iron (as BHT and TBHQ did – discussed below).

The activity of propyl gallate was found dependent on the pH of the liposome dispersion (measured for pH range 2 – 6). Propyl gallate was effective in inhibition of LMW iron-mediated oxidation at pH > 3.5 (with a complete inhibition at pH 5 – 6), while at pH < 3.5 propyl gallate turned into a strong prooxidant, increasing the OUR rates by up to 400% (**Figure 35**). Since the pH of all the liposome dispersions and emulsions in this thesis was above 3.5 this phenomenon was not of concern. It might however be relevant for stomach conditions where pH decreases down to 1.

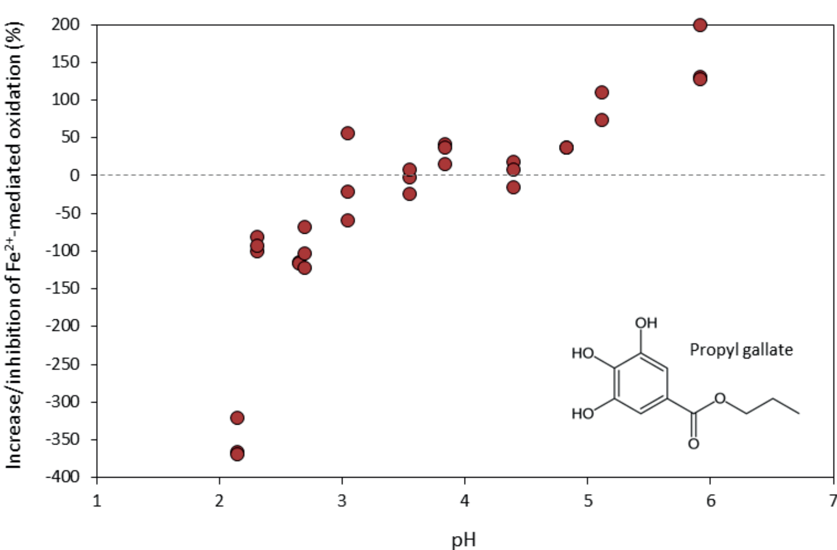

Figure 35 A relative effect (% increase or % inhibition) of propyl gallate (50 μM) on Fe^{2+}-mediated lipid oxidation (10 μM Fe^{2+}) in 0.6% liposomes at different pH. Each point represents one measurement.

The first choice as a positive control was however BHT due to its high antioxidant efficiency in bulk lipids and indifference to iron, since the BHT molecule does not have any functional groups for binding metals. The effect of BHT (5 – 50 μM) on LMW iron-mediated oxidation is shown in Figure 36 (experimental conditions are described in the figure caption). BHT inhibited both Fe^{2+}- and Fe^{3+}-mediated oxidation, and did not affect the equilibrium drop in Fe^{2+}-mediated oxidation. This confirmed that BHT did not interact with iron (such as caffeic acid, ascorbic acid or chelators), and only the radical scavenging abilities are involved in the inhibition mechanism.

The oxygen uptake consumption after the addition of LMW iron to the liposome dispersion containing BHT did not follow the expected linear function (as seen for controls). The oxygen uptake rate was gradually reduced giving non-linear oxygen concentration curves, which made quantification of the inhibition effects problematic. For this reason, BHT was discarded as a positive control.

The non-linear decrease in oxygen consumption is unclear at the moment. One possible explanation could be that scavenging of lipid free radicals by BHT happens much faster than production of the radicals in the red-ox cycling of iron and autoxidation.

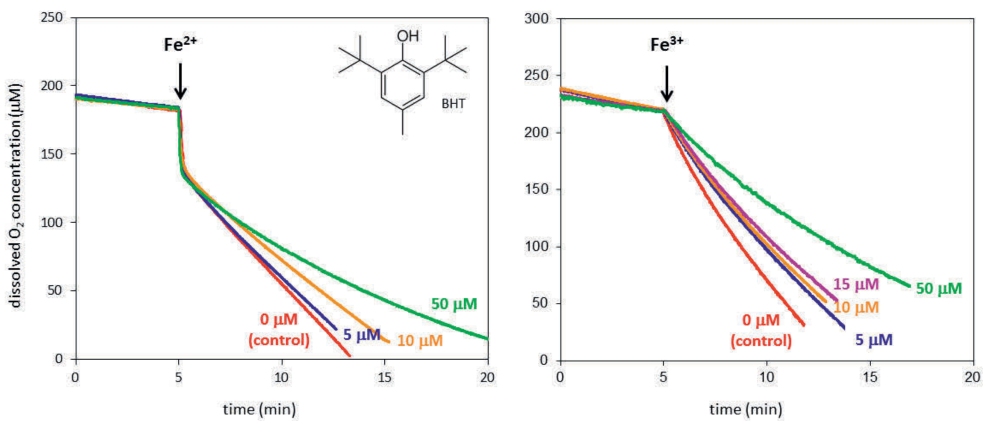

Figure 36 Oxygen uptake curves for Fe^{2+}-mediated oxidation **(left plot)** and Fe^{3+}-mediated oxidation **(right plot)** in 0.6% liposomes (10 μM Fe, pH 5.0, 37°C) and for different concentrations of butylated hydroxytoluen (BHT) (5, 10, 15 and 20 μM) in the liposome dispersion.

Another alternative for a reference compound was *tert*-butylhydroquinone (TBHQ). When Fe^{2+} was added to the liposome dispersion containing TBHQ, an increase rather than decrease in oxygen consumption was observed, *i.e.* TBHQ behaved as a prooxidant in combination with LMW iron. The oxygen consumption did not follow a linear function (similarly to BHT) which complicated quantification of the prooxidant effect (Figure 37). Therefore, TBHQ was discarded as a reference compound as well.

The pro-oxidative behaviour of TBHQ could be attributed to a reduction of Fe^{3+} to Fe^{2+}, assuming that more than one TBHQ molecule would participate in this conversion, since TBHQ does not have a chelation site for metals (as caffeic acid and ascorbic acid have). The prooxidant mechanism of TBHQ was not investigated further in this study since synthetic antioxidants were not of primary interest.

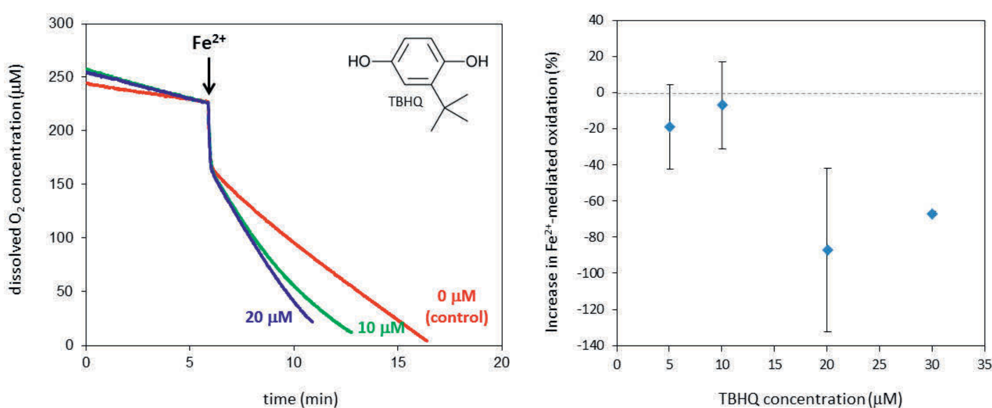

Figure 37 Representative oxygen uptake curves for Fe^{2+}-mediated oxidation in 0.6% liposomes (15 μM Fe^{2+}, pH 5.2, 37°C) containing, 0, 10 and 20 μM TBHQ **(right plot)** and relative increase (%) of the Fe^{2+}-mediated oxidation caused by TBHQ (5, 10, 25 and 30 μM) in the liposomes. The values are average values ± standard deviation of 2 to 6 parallels **(left plot)**.

5.5 Zeta-potential in liposomes containing iron

In relation to the association of LMW iron with the negatively charged heads of phospholipids (proposed in **PAPER I**), a series of zeta-potential measurements in liposomes was performed to evaluate whether this association could be seen on the changes of zeta-potential, which to a certain degree reflects the surface charge of lipid droplets. It was hypothesized that LMW iron attached to the phospholipid interface would decrease the negative zeta-potential of the liposome vesicles.

Zeta-potentials were measured in fresh 1.5% liposomes at three different pH values (3.5, 5.5, and 7.0). The effect of Fe^{2+} on the zeta-potential was evaluated by comparisons of zeta-potentials in liposomes with and without Fe^{2+}. For liposomes without Fe^{2+} only the carrier of Fe^{2+} was added, *i.e.* diluted HCl solution (blank). The results are given in Figure 38.

The results however differ with the pH: at pH 3.5 the presence of Fe^{2+} significantly decreased the zeta-potential, the opposite effect was observed at pH 5.5, and no effect was observed at pH 7.5. It is an open question whether the measurement of zeta-potential is a good enough technique for proving/disproving the hypothesis, since the technique measures the charge near the surface of lipid particles, but not directly at the surface.

Nevertheless, low pH favours iron dissolution in aqueous media which would improve the total iron content, and reduced the negative charge of the liposomes. This would inhibit the electrostatic attraction between iron and the liposome surface, but could also favour penetration of iron ions deeper into the phospholipids heads, which could decrease the overall zeta-potential.

The highest iron activity was observed at pH 4.5 – 5.5 (**PAPER I**). Therefore it was expected that the change in zeta-potential would be highest at pH 5.5. An even more negative zeta-potential (by 3.4 units) was measured in the liposome dispersion containing Fe^{2+} in comparison to dispersion to dispersion without Fe^{2+}. At the moment, we do not a clear explanation for this phenomenon. No change in the zeta-potential was observed at pH 7.0. Iron tends to precipitate at pH > 5.5 due to instability in neutral and basic environment. This could explain the stable zeta-potential since only a fraction of the added iron would interact with the particle surface.

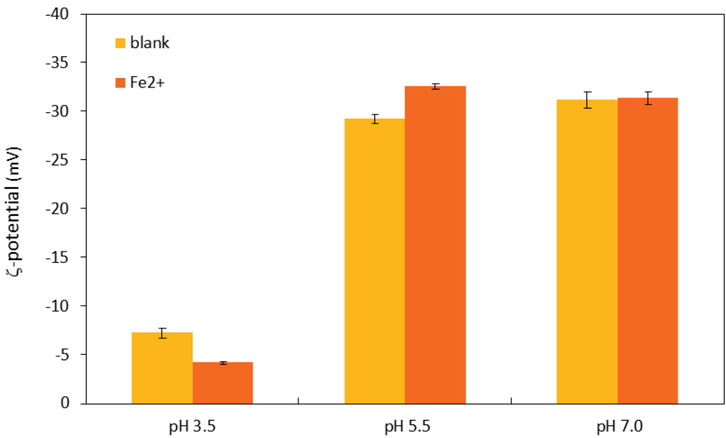

Figure 38 Zeta-potential values in 1.5% liposomes containing LMW iron (10 μM Fe^{2+}) at different pH of the liposomes. The values are average values ± standard deviation of three subsequent determinations performed at 30°C.

6 Results and discussion

Low molecular weight (LMW) iron- and methemoglobin (metHb)-mediated peroxidation of marine lipids in emulsions stabilized with phospholipids and in liposomes was the main focus of this thesis. The impacts of several physicochemical and chemical factors, including dietary antioxidants and gastric juice, on LMW iron- and metHb-mediated oxidation were determined.

The key results and outcomes are highlighted in the following sections.

6.1 Iron- vs methemoglobin-mediated oxidation

Continuous recordings of dissolved oxygen consumption in the liposome dispersions and quantification of the oxygen uptake rates revealed major differences in the prooxidant activity of LMW iron and methemoglobin (metHb).

LMW iron behaved as a lipid oxidation catalyst (**PAPER I**), while the prooxidant activity of metHb decreased over time eventually reaching near zero-activity, which was manifested as an exponential decrease in the dissolved oxygen concentration (**Additional data**). The activity of a freshly introduced prooxidant into the liposome dispersion was shown to be much higher for methemoglobin than for an equimolar level of LMW iron. In this view, metHb is a potent mediator of lipid-oxidation but is not a true catalyst.

The decrease in the prooxidant activity of metHb over time observed in this study correlates well with findings of Grunwald *et al.* [155] The authors proposed that destruction of the heme-group by lipid hydroperoxides during lipid oxidation, which might in addition release free iron into the system, decreased the activity of metHb.

In order to hinder metHb mediated oxidation, the antioxidant strategies should aim at i) deactivation of released LMW iron by chelators, and ii) inhibition of metHb activity by reducing compounds, such as ascorbic acid, and iii) scavenging of lipid radicals generated by metHb-mediated oxidation during the active period of metHb (**Additional data**).

6.2 Properties of emulsifier

An emulsion is composed of three distinct phases: 1) aqueous phase, 2) interphase, and 3) lipid phase (core of the droplet). The majority of emulsions in this thesis had the interphase composed of phospholipids (PL) – these were both of marine (cod, herring) and non-marine (soy, bacteria) origin. Some emulsions were prepared with Tween 20 (**PAPER I**). The droplet core contained fish oil.

The nature of the interphase is considered one of the key factors for the oxidative stability of emulsions [53, 54]. Phospholipids are categorised as an anionic emulsifier, while Tween 20 is a neutral

emulsifier. Both substances are likely to contain some level of pre-formed (endogenous) lipid hydroperoxides (LOOH) [57]. In the phospholipids used in this thesis various levels of pre-formed LOOH were determined, ranging from 1.6 ± 0.2 mmol LOOH/kg (bacterial PL) to 37.6 ± 1.1 mmol LOOH/kg (herring PL) (Table 7).

The presence of pre-formed LOOH in the phospholipids was shown to be of crucial importance for the prooxidant activity of both LMW iron (**PAPER I**) and methemoglobin (**Additional data**), which verified the pro-oxidative mechanisms, *i.e.* decomposition of lipid hydroperoxides by free iron and heme-iron, respectively. These pre-formed LOOH were not a limiting factor for the decomposition, given their abundance in relation to LMW iron and metHb concentration, and given the abundance of unsaturated lipids to sustain the red-ox cycling of iron and activity of metHb.

Tween 20 was shown to be a protective emulsifier in 1.5% cod liver oil emulsions. After the endogenous LOOH in Tween 20 were broken down by Fe^{2+}, the oxygen uptake rates were not significantly increased as a result of iron-mediated oxidation of the oil in the core of the droplets. This was consistent for different concentrations of Tween 20 (2.5 – 15.0% w/w) in the emulsion (Figure 7 in **PAPER I**).

Phospholipids in emulsions and liposomes are shown to attract both LMW iron and methemoglobin, which leads to acceleration of lipid oxidation (**PAPER I – III, Additional data**) by facilitation of the pro-oxidative mechanisms of the two prooxidants.

The degree of unsaturation of the fatty acids in the phospholipids is shown to have an impact on the rate of iron-mediated oxidation. The use of soy and bacterial phospholipids, which contain much lower levels of PUFA than the marine phospholipids (Table 7), lead to lower oxygen uptake in iron-mediated oxidation (Figure 4C in **PAPER I**). This was attributed to the lower proportion of oxidisable substrate (double bonds) after the pre-formed LOOH were decomposed.

The amount of the phospholipid emulsifier (0.5 – 15.0% w/w) influenced the oxidation rates in iron-mediated oxidation both positively and negatively. It is shown that 5 – 10% (w/w lipid base) of emulsifier results in the highest oxygen uptake rates in iron-mediated oxidation (Figure 7 in **PAPER I**). This was found due to a formation of smaller droplets with increasing amount of the emulsifier.

The attraction between phospholipids and iron ions is discussed in **PAPER I**. It is proposed that iron ions are, at least partially, retained by the phosphate groups of the phospholipid heads. Such location may enable necessary contact between pre-formed LOOH on PUFA which are situated deeper in the phospholipid interphase. The location can also create competition reactions for iron precipitation at pH > 5 and iron chelation by various compounds dissolved in the aqueous phase, as demonstrated with xanthan gum (further commented in Section 6.5). A 3D-illustration of a peroxidesed and coiled DHA within a choline molecule and iron atom embedded in the phospholipid plane is shown in Figure 39.

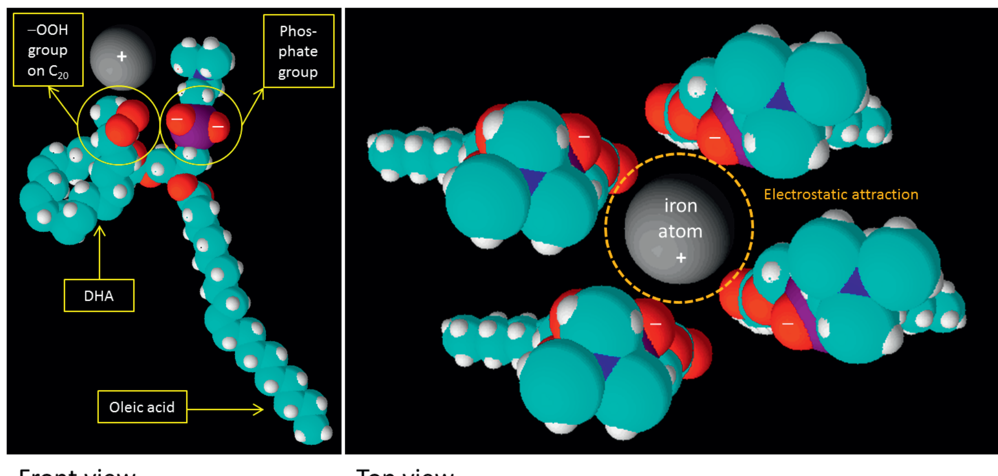

Figure 39 Left: A front view of phosphatidyl choline bearing oleic acid and coiled docosahexaenoic acid (DHA), with a pre-formed hydroperoxide group (–OOH) located at C_{20} on the DHA chain, and an iron atom (to scale). **Right:** A top view of four phosphatidyl choline molecules assembled in a plane, with a positively charged iron atom embedded in between the negatively charged phosphate groups of the phospholipid heads. The atom sizes are to scale (created with ACD/ChemScatch).

6.3 Droplet particle size

The total surface area of the droplets was found of importance for LMW iron-mediated lipid oxidation (**PAPER I**). It was demonstrated that smaller droplets (liposome vesicles made of phospholipids) were more prone to oxidaiton than 100-fold larger droplets (emulsion droplets stabilised with phospholipids). Since iron-mediated peroxidation is facilitated by encounters between the iron ions and the pre-formed LOOH, larger overall areas, *i.e.* smaller droplets, give higher likelihood of these encounters.

In this view, LMW iron-mediated oxidation can be seen as a phenomenon occurring on the droplet surface as both the LMW iron and LOOH are associated with the phospholipid interface. Therefore, the rates in LMW-iron mediated oxidation will be proportional to the surface area, given a large excess of pre-formed LOOH in the interphase, and the rates will be dependent on the total surface area, which is given by number of droplets and their size.

6.4 pH of the aqueous phase

The acidity (pH) of the aqueous phase affects the charge of the droplets and the level of Fe^{2+} and Fe^{3+} dissolution [12]. It was shown that the most favourable pH for LMW iron activity lies in the interval 4.5 – 5.5 (Figure 40) where the balance between iron attraction by the droplets and iron precipitation is most optimal (**PAPER I**).

LMW iron was to some degree catalytically active in the pH range 5.5 – 7.0 where precipitation of Fe^{3+} was expected, which would completely prevent LMW-iron mediated oxidation, due to low stability of iron at neutral pH. Therefore, it was suggested that iron ions retained by the phospholipid heads become unavailable for the precipitation reactions, and the association of iron with the phospholipid heads enables iron-mediated oxidation even at more neutral pH.

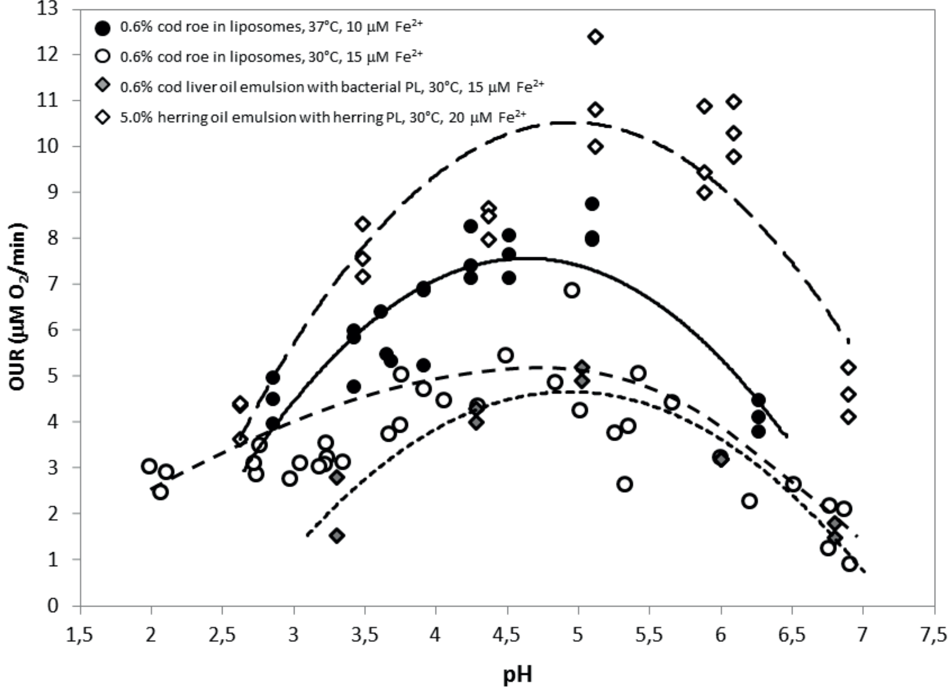

Figure 40 Influence of pH on Fe^{2+}-mediated oxidation in different emulsions and liposomes. Each point represents a single measurement (figure retrieved from **PAPER I**).

6.5 Dissolved compounds

A positive correlation between the content of endogenous chloride anions (Cl^-) and i) a decrease in oxygen uptake rates and ii) a tendency to neutralize the negatively charged liposome vesicles and emulsion droplets was found (**PAPER I**). These effects were however attributed to the counter ions accompanying Cl^- (*e.g.* Na^+, K^+, Mg^{2+}) rather than to the chloride anions. The cations may favourably modify the droplet charge making it less electrostatically attractive for iron ions. Nevertheless, sodium chloride (NaCl) added to an emulsion was demonstrated to inhibit iron-mediated oxidation in a concentration dependent manner (Figure 9 in **PAPER I**).

A thickener, xanthan gum (0.2%), dissolved in the aqueous phase of 10% emulsions inhibited iron-mediated oxidation (Figure 8 in **PAPER I**). This was attributed to the chelating abilities of xanthan gum [162]. The quantity of xanthan gum added to the emulsion was however expected to chelate all the added iron which would inhibit iron-mediated oxidation completely. Since only partial inhibition was observed, it was concluded that there might be competition reactions between retention of LMW iron by phospholipid heads and by xanthan gum.

6.6 Antioxidants

An addition of antioxidants into food containing marine LC omega-3 PUFA is one of the most common approaches to protect the healthy omega-3 lipids from oxidation and to increase the oxidative stability of the food. Therefore, several dietary antioxidants were used in this thesis and their effect on LMW iron- and meHb-mediated oxidation was evaluated (**PAPER II**, **PAPER III** and **Additional data**).

6.6.1 Phenolic acids

The effect of phenolic acids (caffeic acid, ferulic acid, and *p*-coumaric acid) on oxidation of 1.5% (w/v) liposomes (pH 5.5) mediated by LMW iron and metHb was evaluated (**PAPER II**).

The activity of phenolic acids was shown to be dependent not only upon the chemical structures of the molecules, but also upon the type of prooxidant in the system. Caffeic acid showed a strong pro-oxidative behaviour in the presence of iron ions, but behaved as an antioxidant in the presence of methemoglobin. The ability to reduce free Fe^{3+} to the more catalytically active Fe^{2+}, *i.e.* to enhance the rate limiting reaction in LMW iron-mediated oxidation, was attributed to the pro-oxidative behaviour of caffeic acid (depicted in Figure 41). The magnitude of the pro-oxidative activity of caffeic acid was shown to be both concentration and pH dependent.

Since caffeic acid showed a dual effect, depending on the type of prooxidant in the system, the activity of caffeic acid was also evaluated in iron-mediated peroxidation in emulsion (**PAPER III**) and in metHb-mediated oxidation in a liposome dispersion (**Additional data**). The same dual effects as in **PAPER II** were observed. Therefore, reducing abilities of caffeic acids seems to be responsible not only for the prooxidative effects, due to reduction of LMW Fe^{3+} to the more prooxidatively active Fe^{2+}, but also for antioxidant effects, due to reduction of hyper active ferryl-Hb species. Further investigation is needed to elucidate the interactions of caffeic acid with heme-proteins and the antioxidant mechanism causing the inhibition effect.

6.6.2 Propyl gallate

Propyl gallate showed excellent inhibition effects in both iron- and metHb-mediated oxidation in 1.5% lipoosmes (pH 5.5) (Figure 3 and Figure 7 in **PAPER II**) and exceeded the performance of phenolic acids. Good inhibition effects of propyl gallate on iron-mediated oxidation were also observed in 10% emulsion (**PAPER III**). Propyl gallate inhibited both the net oxygen uptake rates and the equilibrium drop in Fe^{2+}-mediated oxidation. Both iron chelation along with scavenging of lipid free radicals in the phospholipid interphase were attributed to these effects.

However, caution should be taken when adding propyl gallate to lipid systems with a low pH (pH < 3.5). At these conditions propyl gallate was shown to be prooxidative in the presence of LMW iron (**Additional data**), showing that the activity of propyl gallate is also dependent on the pH of the aqueous phase.

6.6.3 Chelators: EDTA and citric acid

The effects of metal chelators – EDTA (synthetic origin) and citric acid (natural origin) – on LMW iron-mediated oxidation in 10% (w/v) herring oil emulsions stabilized with herring phospholipids at pH 3.5 – 5.5 are shown in **PAPER III**. EDTA and citric acid completely inhibited iron-mediated oxidation when they were added in twice the molar ratio to iron. The activity of the chelators was in accordance with ionization of –OH group (given by pKa values) showing that as long the groups were partially or fully ionised (pH > 3.5) the compounds showed chelating abilities.

EDTA was used for deactivating endogenous metals and iron released from methemoglobin in the research on the activity of meHb. The data in this thesis indicate that EDTA was capable of slight inhibition of metHb-mediated oxidation (by 33%) (**Additional data**). At this point, the mechanism by which EDTA affects the activity of methemoglobin is not clear.

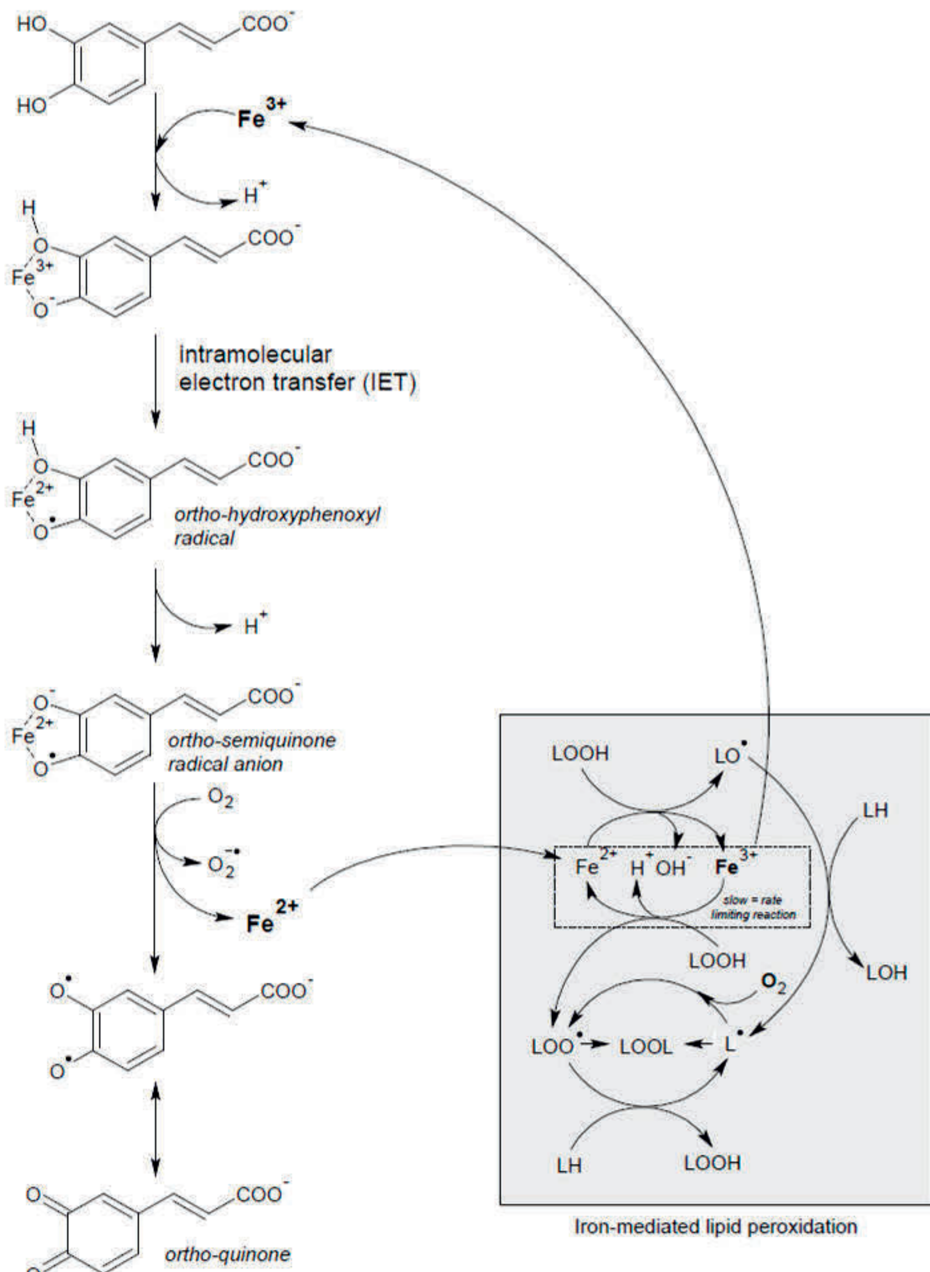

Figure 41 The intramolecular electron transfer of Fe^{3+} to Fe^{2+} by caffeic acid and the linkage to iron-mediated lipid peroxidation (figure adapted from **PAPER II**)

6.6.4 Tocopherols

α-Tocopherol enhanced lipid oxidation catalysed by LMW iron in emulsions (**PAPER III**), but it inhibited metHb-mediated oxidation in liposome dispersion (**Additional data**). It was shown that deactivation of LMW iron by EDTA prevented the prooxidant effect of α-tocopherol. Therefore the role of α-tocopherol as a prooxidative synergist, acting by reduction of Fe^{3+} to the more active Fe^{2+}, was confirmed.

Both α-tocopherol and δ -tocopherol were efficient in inhibition of metHb-mediated oxidation. The inhibition effect was slightly higher for δ-tocopherol than for α-tocopherol **(Additional data)**. The inhibition of metHb-mediated oxidation is believed to be due to scavenging of lipid radicals in the phospholipid interface.

These results suggest that the final effect of α-tocopherol is determined not only by the concentration of tocopherol, but also by the extent of reactions with LMW iron and with lipid radicals – reactions which may run simultaneously.

6.6.5 Ascorbic acid and ascorbyl palmitate

Oxygen uptake curves in LMW iron-mediated oxidation in emulsions containing ascorbic acid revealed that ascorbic acid was depleted by interactions with iron. Unfortunately, it was not possible to determine from the oxygen uptake curves whether reducing abilities of ascorbic acid were responsible for removal of dissolved oxygen in co-operation with Fe^{2+} or for reduction of Fe^{3+} to Fe^{2+}, *i.e.* acceleration of iron-mediated oxidation (**PAPER III**).

Ascorbic acid was capable of decreasing the prooxidative activity of α-tocopherol despite the presence of LMW iron (**PAPER III**). It was concluded that the mutual reactions between LMW iron, ascorbic acid, and α-tocopherol will determine the final effect and final rates of lipid peroxidation.

Ascorbic acid was efficient in inhibiting metHb-mediated oxidation in liposome dispersions in a concentration dependent manner (**Additional data**). A lipid soluble analogue of ascorbic acid, ascorbyl palmitate, was also efficient in inhibiting metHb-mediated oxidation in dependence on its concentration (**Additional data**).

Due to the interactions with iron leading to increased consumption of dissolved oxygen, ascorbic acid was a useful tool for indirect evaluation of whether the liposome/emulsion system contains endogenous metals (**Additional data**).

6.6.6 Carotenoids: Astaxanthin and β-carotene

Lipid soluble carotenoids, astaxanthin and β-carotene, showed only minor effects on LMW iron-mediated lipid oxidation in emulsions (**PAPER III**) which could be due to low concentrations or low proportion in the emulsion interphase. Nevertheless, these results suggest that the two compounds were indifferent to LMW iron itself. Astaxanthin inhibited metHb-mediated oxidation in liposomes in a concentration dependent manner (**Additional data**), showing the ability to scavenge lipid free radicals in the phospholipid interface.

6.6.7 Effects of antioxidants: Conclusion

The results in this thesis shows that interactions between LMW iron and selected antioxidants (caffeic acid, α-tocopherol, ascorbic acid) negatively affects the rates of lipid peroxidation in fish oil emulsions stabilized with phospholipids rich in LC omega-3 PUFA. These interactions lead to reduction of Fe^{3+} to the more catalytically active Fe^{2+}, and therefore these antioxidants show prooxidative effects in the presence of LMW iron. This is manifested by increased oxygen uptake consumption (Figure 42, and **PAPER II, III**). Not only these interactions enhance lipid oxidation, but they also deplete the antioxidants, since the antioxidants act as reactants in the reduction processes.

The final oxidation rate in the system is therefore determined by the mutual reactions between antioxidants-iron, iron-lipids, and lipids-antioxidants, and the concentration of each substance. Such a triangle of dependency was observed for the combination of ascorbic acid/α-tocopherol/LMW iron/phospholipids (**PAPER III**). The interactions of antioxidants with LMW metals must therefore be considered when interpreting the activity/behaviour of antioxidants in multiphase systems.

On the other hand, no prooxidant effect of the tested antioxidants (caffeic acid, ascorbic acid, ascorbyl palmitate, astaxanthin, and α- and δ-tocopherol) was detected in metHb-mediated oxidation. The antioxidants inhibited metHb-mediated oxidation by various degrees and, in addition, in dependence on the concentration and ratio to metHb.

In conclusion, the type and quantities of prooxidants in lipid systems should be known to develop effective protective strategies against lipid oxidation for the system, using antioxidants.

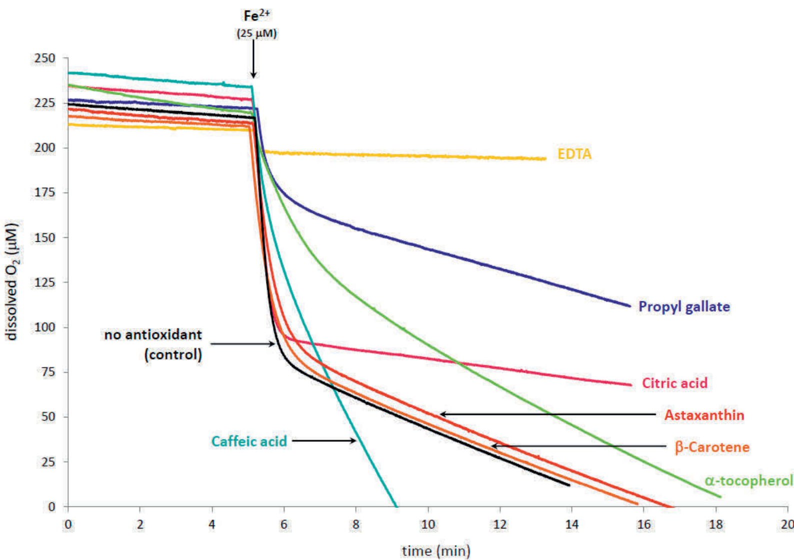

Figure 42 Direct comparison of representative oxygen consumption curves in 10% herring oil emulsion stabilized with herring phospholipids and containing α-tocopherol, β-carotene, astaxanthin, propyl gallate, caffeic acid (each at 100 μM), EDTA or citric acid (each at 50 μM), and no antioxidant (control) in Fe^{2+} (25 μM)-mediated oxidation, illustrating the effects of the antioxidants on LMW-iron mediated oxidation (figure retrieved from **PAPER III**).

6.7 System properties as oxidation hurdles

The outcomes on the impact of individual factors on lipid oxidation in emulsions and liposome dispersions rich in LC omega-3 PUFA clearly show that multiple factors affect the oxidation rates (the factors which were studied in this thesis are schematically illustrated in Figure 14). It is clear that all these factors operate simultaneously. By careful manipulation of the system properties and choice of antioxidants, it could be, to a certain degree, possible to use the individual factors, as hurdles for LMW iron- and metHb-mediated lipid oxidation in emulsified systems.

The acidity of the aqueous phase (pH) is shown to be a significant modulator of lipid oxidation rates, since it affects several factors: solubility of ionic iron, ionisation of metal chelators, charge of emulsion droplets' surface, *i.e.* the electrostatic attraction towards positively charged iron ions, and the radical scavenging and reducing abilities of phenolic antioxidants. Therefore, alterations in pH could improve oxidative stability of more complex emulsions.

The unsaturation level of phospholipids located in the interphase of an emulsion is shown to reduce the oxidation rates in LMW iron-mediated oxidation. Therefore, the choice of less

unsaturated phospholipids as en emulsifier may help to decrease the rate of lipid oxidation in the core of the emulsion droplet which, on the other hand, may have a high unsaturation level.

6.8 Antioxidant activity assays

The antioxidant activity assays (Folin-Ciocalteu, FRAP, DPPH, ABTS) used in **PAPER II** for evaluation of the antioxidant efficiency of phenolic acids and propyl gallate predicted propyl gallate and caffeic acid to be the most active antioxidants from the group. The assays however reflected mainly the reducing power of the compounds. This results in a misleading interpretation of the antioxidant abilities and does not predict the behaviour in lipid systems. As demonstrated on caffeic acid, the antioxidant activity assays did not reveal the prooxidative interactions with iron which turned the compound into a prooxidant.

Therefore, application of the predications from the antioxidant capacity assays on lipid systems, and systems where prooxidants are presents, may not reflect the real behaviour of the antioxidants in the lipid system.

6.9 Post-prandial lipid oxidation

The stomach is an entry organ for marine lipids in food and can be seen as an intermediate station, before the food is further metabolised in the gastrointestinal tract. Therefore, **PAPER IV** evaluates whether emulsified marine lipids can get oxidised in acidic stomach environment, *i.e.* while they are retained in the stomach, and whether authentic gastric juice has the potential to act as a pro- or anti-oxidative medium. In addition, iron and methemoglobin were added to the system to characterize the effect of the acidic environment on their prooxidative activity. Beverages containing antioxidants were added to assess their effect on lipid oxidation in the acidic environment.

Oxidation of emulsions stabilized with phospholipids and liposomes, both containing marine lipids, was followed in *in vitro* digestion models containing authentic human gastric juice, and compared to models containing hydrochloric acid solution (HCl). The oxidation was followed by measuring peroxide value (PV), concentration of thiobarbituric acid reactive substances (TBARS) and oxygen uptake rate (OUR) in the model systems during 2.5 h incubation at pH 4 and 37 °C in darkness.

No difference between oxidation in gastric juice and HCl solution was found in both liposomes and emulsion, which suggests that it is lipid system properties rather than gastric juice itself that determine the degree of oxidation. PV, TBARS and OUR increased during the incubation which suggests that acidic environment of stomach is not preventive in respect to lipid oxidation. However,

gastric juice reduced the prooxidant activity of added LMW iron, but did not reduce the activity of added methemoglobin (measured by the oxygen uptake method).

Berry juice, green tea, red wine, and caffeic acid reduced the OUR in the acidic environments while coffee, ascorbic acid and orange juice increased the rates. The study concludes that beverages accompanying foods containing marine lipids will affect the course of post-prandial lipid oxidation.

6.10 Activity of caffeic acid in different fish lipid matrices: A review (PAPER V)

PAPER V (additional contribution) is a review paper on the effect of caffeic acid on lipid oxidation in different systems containing fish lipids (bulk fish oils, liposomes from cod roe phospholipids, fish oil emulsions, washed cod mince, regular horse mackerel mince and fish oil fortified fitness bars). The review discusses mechanisms involved in the antioxidative and prooxidative effects of caffeic acid found in the different systems. The paper includes outcomes reported in **PAPER II**.

The data from the different systems show that the antioxidant activity of caffeic acid is dependent on the physical state of the lipids and the composition of the intrinsic matrix in which the lipids are situated.

Caffeic acid prevented rancidity in both unwashed and washed fish mince, the latter system fortified with hemoglobin. In unwashed minces, the activity was however clearly dependent on the lipid to antioxidant ratio. In these systems, a redox cycle between caffeic acid and the endogenous reducing agents, such as ascorbic acid and tocopherols, were further thought to play an important role for the antioxidant effects. The effect of caffeic acid was also highly dependent on the storage temperature, showing higher effectiveness above rather than below 0°C. Caffeic acid was not able to inhibit oxidation in the bulk fish oils, fish oil-in-water emulsions and fish-oil enriched fitness bar. In the liposome system, caffeic acid inhibited hemoglobin-mediated oxidation but strongly promoted Fe^{2+}-mediated oxidation.

The review concludes that caffeic acid can significantly prevent hemoglobin-mediated oxidation in fish muscle foods but its activity in food emulsions and liposomes is highly dependent on the pH, the type of emulsifier, and the prooxidants present.

6.11 Evaluation of oxygen uptake measurements

Oxidation of LC omega-3 fatty acids in emulsions stabilised with phospholipids and in liposome dispersions was followed by recording the dissolved oxygen concentration in the systems, which is a less common approach in studies on lipid oxidation.

The measurement of dissolved oxygen consumption is shown to be a robust tool for monitoring lipid peroxidation in the two systems. As shown in **PAPER IV**, it may also serve as a complimentary method to the conventional determinations of primary and secondary oxidation products.

By quantification of the rates of consumption of the dissolved oxygen, it was possible to screen the impact of different factors on both LMW iron and metHb lipid oxidation individually and in a controlled way. Large collections of data are necessary for the development of mathematical models which predict lipid oxidation. Since the oxygen uptake measurement is relatively quick to prepare and perform, it has the potential for screening and modelling of lipid oxidation in systems rich in LC omega-3 PUFA, including the effects of physicochemical and chemical variables and the presence of pro- and antioxidants.

The oxygen uptake method is especially useful for monitoring of the oxygen consumption kinetics and how the kinetics is affected by the different factors. This approach is very useful for testing the effects of different antioxidants on the prooxidant activity of LMW iron and metHb and for evaluation of the interactions between LMW iron and the antioxidants.

As shown in **PAPER IV**, the method may also be beneficial for a quick assessment of the effects of different substances (in this case, antioxidant rich beverages) on oxidation in a specific system (in this case, a digestion mixture of lipids and gastric juice). This example may further broaden the spectrum of applications for the oxygen uptake methodology.

Continuous recordings of oxygen uptake have however limitations when it comes to adequate quantification of the oxygen uptake rates, as the oxygen consumption rate may not be constant during a measurement (as shown in metHb-mediated lipid oxidation). This was the main limitation in the method, which could be overcome by better and more advanced mathematical processing of the oxygen uptake curves.

7 Concluding remarks

This thesis investigates low molecular weight (LMW) iron- and methemoglobin (metHb)-mediated peroxidation of marine long chain omega-3 polyunsaturated fatty acids in emulsions stabilized with phospholipids and in liposome dispersions, representing food-related lipid systems. The impacts of several physicochemical and chemical factors, including dietary antioxidants and gastric juice, on LMW iron- and metHb-mediated oxidation were determined.

This study shows that the overall rate of LMW iron-mediated oxidation in the two systems is given by a combination of multiple factors. The following factors were investigated in this thesis: pH of the aqueous phase and pH dependent droplet charge, saturation level and concentration of the phospholipid emulsifier, emulsifier concentration dependent droplet size, and concentration of water soluble components, namely sodium chloride (salt) and xanthan gum. When optimally set, each of these factors may represent a system specific hurdle for LMW iron-mediated lipid oxidation in emulsified systems.

Methemoglobin was shown to be a stronger promoter of lipid oxidation in comparison to LMW iron but not a true catalyst, in contrast to LMW iron which behaved as a lipid oxidation catalyst. The activity of a freshly introduced prooxidant was shown to be much higher for methemoglobin than for LMW iron, but the activity of methemoglobin decreased over time. These findings suggest that metHb-mediated oxidation might be relevant for early oxidation of emulsified systems (such as production phase). In later stages (such as storage), meHb-mediated oxidation is likely to be replaced by slower but persistent oxidation mediated by LMW iron – both endogenous iron and iron released from methemoglobin. Antioxidant strategies should therefore aim on deactivation of both methemoglobin and LMW iron simultaneously.

This study further demonstrates that the effects of dietary antioxidants are dependent on the type of prooxidant in the system. Caffeic acid and α-tocopherol interacted with LMW iron, which resulted in acceleration of peroxidation. None of the tested antioxidants turned into a prooxidant in metHb-mediated oxidation. EDTA (metal chelator) was highly efficient in chelating LMW iron but had a low effect on the prooxidant activity of metHb. Therefore, direct interactions of antioxidants with LMW iron, which do not result in metal chelation, should be prevented to assure that antioxidants will not aid prooxidative effects. The outcomes of this thesis underline that for correct assessments of the effects of antioxidants in emulsified systems, the type and concentration of the prooxidant in the system must be known.

Finally, this study shows that the authentic human gastric juice neither prevents nor promotes oxidation of emulsified marine lipids, in comparison to hydrochloric acid solution. No effect of gastric

juice on the activity of methemoglobin was found, but gastric juice lowered the prooxidant activity of LMW iron to a certain degree. It is also shown that post-prandial oxidation of marine lipids may be altered both positively and negatively by antioxidant rich beverages. Based on the research in this thesis, consumption of LC omgeag-3 PUFA supplements (*e.g.* fish oil capsules, bulk fish oil) together with iron and multivitamin supplements, the latter usually containing reducing agents (namely, ascorbic acid), is not recommended, due to prooxidant behaviour of free iron alone and in combination with ascorbic acid, and a limited ability of acidic stomach environment to reduce the prooxidative effects.

Lipid oxidation in the lipid model systems was followed by continuous monitoring of consumption of the dissolved oxygen by unsaturated fatty acids. In other words, the essential lipid oxidation substrate – oxygen – was in the focus of the measurements. This is a different approach than in the majority of published studies on lipid oxidation in which mainly lipid oxidation products are determined.

This thesis shows that the method is useful for the assessment of oxygen consumption kinetics and oxygen uptake rates in liposome dispersions and emulsions stabilized with phospholipids. Both the inherent oxygen uptake in the systems and the oxygen consumption after addition of prooxidants can be measured in the frame of one experiment and in a relatively short time, which makes the method advantageous for studying lipid oxidation.

Oxygen uptake kinetics in LMW iron- and metHb-mediated oxidation in liposomes and emulsions is presented in this thesis. Alterations in the oxygen uptake caused by the added antioxidants and variations in the physicochemical and chemical factors were directly reflected in both the oxygen consumption kinetics and the net oxygen uptake rates calculated from the oxygen consumption curves. The method is shown to be robust with respect to the lipid system.

Therefore, the oxygen uptake method has the potential to be used as a tool for screening, modelling and verification of system conditions and factors affecting lipid oxidation in emulsion-like systems containing unsaturated lipids. The method can give additional information on the oxidation processes in these systems and serve as a complimentary tool for assessment of lipid oxidation by the conventional methods determining lipid oxidation products.

8 Recommendations for future work

The field of oxidation of marine lipids is enormous and many aspects remain to be clarified. In relation to the prevention of lipid oxidation in food emulsions enriched with long chain omega-3 polyunsaturated fatty acids, the following aspects could be investigated by means of the oxygen uptake method:

Measurements of the dissolved oxygen consumption in real food emulsions (*e.g.* dairy products, beverages, minces and homogenates, etc.) could bring new insights into the autoxidation, as well as into oxidation mediated by different prooxidants in these systems, and especially into the kinetics of the oxygen consumption in these systems. A systematic investigation of the systems' conditions could uncover key hurdles for inhibiting lipid oxidation in a particular system, and effective combinations of antioxidants for each system could be found.

The prooxidant activity of methemoglobin needs to be further investigated – for instance with respect to the release of hemin and free iron, pH of the environment, dissolved compounds, and physicochemical properties of the system. The prooxidant activity of different methemoglobins (*e.g.* from fish and aquatic animals *vs* mammals) as well as other prooxidative metals (such as copper) could be evaluated by means of the oxygen uptake measurements to elucidate their pro-oxidative mechanisms.

Different natural antioxidants individually and in mixtures, plant/herbs/algal extracts rich in natural antioxidants and novel antioxidants (such as peptides and phenolipids) should be tested against autoxidation and both iron- and methemoglobin-mediated oxidation. Using the model systems of liposome dispersions and emulsions and food emulsions, combined with oxygen uptake measurements would be a more realistic approach, rather than using lipid-free antioxidant capacity assays. This would help to assess the antioxidant potential and antioxidant power of these substances more correctly. Synergistic or antagonistic relations could be discovered, as well as interactions with prooxidants. Optimal conditions for the activity of the antioxidants in specific emulsified system, *e.g.* in relation to the pH of the environment, could be investigated by the oxygen uptake method.

In addition to testing different antioxidants, different emulsifiers (SDS, Tweens, Citrems, proteins, Brijs and others) should be investigated in iron- and methemoglobin-mediated oxidation in fish oil emulsions. Characterization of the effects of the different emulsifiers on the activity of the two prooxidants by the oxygen uptake method could help to increase the knowledge on the impact of emulsifiers on iron- and methemoglobin-mediation and on oxidative stability of emulsions.

Systematic studies and large data sets are necessary for thorough understanding, predicting and modelling of lipid oxidation. The oxygen uptake method could be used for a systematic investigation of factors affecting lipid oxidation, including antioxidants, in different lipid systems rich in LC omega-3 fatty acids. Data from such measurements could be used to establish lipid oxidation prediction models.

The quantification of the oxygen uptake rates in non-linear oxygen uptake curves and the verification of oxygen consumption kinetics need to be improved and more accurate, which could be achieved by mathematical processing of the recorded oxygen uptake curves.

The association of iron atoms and methemoglobin with the phospholipid interphase is an area which has not yet been thoroughly investigated. Clarification of the type and strength of this association and specification of the exact location of the iron atoms and methemoglobin in relation to the phospholipid interphase would help to better understand iron- and methemoglobin-mediated oxidation in emulsified systems stabilized with phospholipids. This would allow designing highly effective strategies for the deactivation of the two prooxidants.

It would be interesting to determine the lipolytic activity and iron-binding capacity of the authentic human gastric juice. A static stomach model, used in the present study, could be replaced by a dynamic model which would better simulate the processes and conditions in the stomach. The extent of post-prandial lipid oxidation in the stomach could be described for a range of marine lipids and products rich in LC omega-3 PUFA, and the effect on lipid oxidation of many more beverages could be investigated (for example white wine, milk, beer and exotic juices). It would be very exciting to study post-prandial oxidation of marine lipids *in vivo*, using human subjects.

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10 Papers

PAPER I

Iron-mediated peroxidation in marine emulsions and liposomes studied by dissolved oxygen consumption

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Research Article

Iron-mediated peroxidation in marine emulsions and liposomes studied by dissolved oxygen consumption

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Low molecular weight (LMW) iron is present in most food in traces or significant amounts. Upon contact with unsaturated lipids LMW iron acts as a prooxidant. This creates oxidative stability problems for products containing marine polyunsaturated lipids. In this work, LMW iron-mediated oxidation in fish oil emulsions stabilized with phospholipids and Tween and in liposomes made from phospholipids was studied. Marine and non-marine sources of phospholipids were used. The aim was to evaluate how physicochemical factors and lipid properties affect the prooxidant activity of LMW iron. The oxidation was followed by measuring the rate of the dissolved oxygen consumption by fatty acids. Red-ox cycling of iron (Fe^{3+}/Fe^{2+}) aided by decomposition of the pre-formed lipid hydroperoxides was the major prooxidation mechanism in both emulsions and liposomes. The elimination of the pre-formed lipid hydroperoxides prevented LMW iron-mediated oxidation. The oxygen uptake rates were highest for various liposomes/emulsions at pH 4–5 making this an optimum pH for iron activity. The pro-oxidant effect of iron was reduced by using less unsaturated phospholipids, specific amounts of emulsifiers, a presence of chloride anions, or xanthan gum. This work suggests that the iron ions are tightly associated with the phosphate groups within the phospholipids heads. The measurement of oxygen consumption is a good tool for systematic oxidation studies in emulsions and liposomes, and may be useful for assessment of optimal conditions for reduction of LMW iron-mediated oxidation in emulsion-like systems.

Practical applications: For a successful addition of marine polyunsaturated lipids into processed food, it is important to understand the pro-oxidation mechanisms of ubiquitous LMW iron, as well as how physicochemical conditions affect the pro-oxidative activity or LMW iron. This work addresses these issues in emulsions and liposomes and the knowledge may help to characterize effective hurdles for pro-oxidant activity of LMW iron. There is a need for quick and inexpensive oxidation assays in order to be able to screen and model the effects of different factors on the oxidative stability of lipids. The measurement of dissolved oxygen in emulsions and liposomes has been used in this study for evaluating the effect of the different psychochemical and chemical factors on iron-mediated oxidation. This method has a potential as a tool for assessment of optimal conditions for prevention of LMW iron-mediated lipid oxidation in emulsion type systems and understanding the possible mechanisms.

Keywords: Emulsion / Iron / Lipid oxidation / Liposomes / Oxygen uptake

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Abbreviations: AV, *p*-Anisidine value; **FID,** flame-ionisation detector; **LC PUFA,** long chain polyunsaturated fatty acids; **LMW,** low molecular weight; **LOOH,** lipid hydroperoxides; **OUR,** oxygen uptake rate; **PL,** phospholipids; **PV,** peroxide value; **SD,** standard deviation; **SE,** standard error; **TBARS,** thiobarbituric acid reactive substances; **TLC,** thin layer chromatography

1 Introduction

Long chain omega-3 polyunsaturated fatty acids (LC PUFA) from marine organisms have a series of important physiological roles in the human body: DHA is mainly incorporated into the phospholipids of cell membranes of for instance the brain and retina [1], eicosapentaenoic acid (EPA) is mainly involved in regulatory functions, such as gene expression or eicosanoid production [2]. LC PUFA are also widely accepted as a part of a healthy diet and in medical treatments [3].

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Despite recommendations issued by a number of authorities, the intake of marine lipids by the modern societies is currently far below the recommended levels [4]. A variety of food products, such as bakery, dairy, and egg products, spreadable fats, meat, infant formulas, and a variety of beverages have therefore been enriched with PUFA and marketed as a way to comply with the recommended intake levels. However, enrichment with marine lipids may lead to changes in flavor, aroma, texture, appearance, decrease the nutritional value, and cause stability and shelf-life problems due to oxidation of the LC PUFA [5].

Lipid oxidation is greatly enhanced by prooxidative compounds, which are ubiquitous in food, either as traces or in significant amounts [6]. The major dietary prooxidants are transition metals, mainly iron (Fe^{2+}/Fe^{3+}) and copper (Cu^{+}/Cu^{2+}), both existing elemental or associated with proteins, such as in hem groups of myoglobin, hemoglobin, enzymes, and pigments [7]. The abundance of iron in food makes its prooxidant activity of particular interest. Heme iron is considered the main prooxidant in red meat and dark muscle fish, while low molecular weight (LMW) iron is the main prooxidant in white muscle fish, and can be found in drinking water, ingredients or packaging materials. LMW iron can be released from the hem groups during food processing, cooking and storage [8].

The pro-oxidant mechanism of LMW iron has been well described in literature [9]. The major pathway is decomposition of preformed lipid hydroperoxides (LOOH) into lipid radicals through red-ox cycling, as shown in Fig. 1. Both ionic

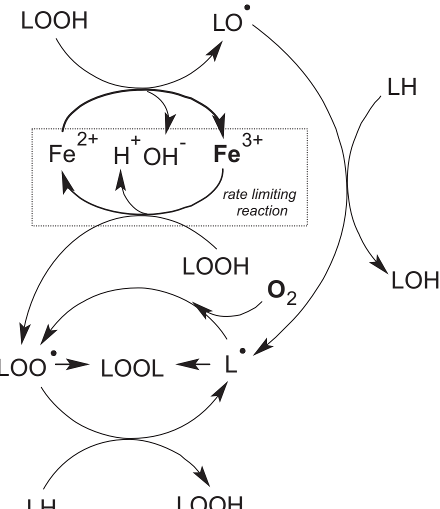

Figure 1. Mechanism for LMW iron-mediated peroxidation of lipids; adapted from [10]. LOOH = lipid hydroperoxide, LOO● = lipid peroxyl radical, LO● = lipid alkoxyl radical, L● = lipid alkyl radical, LH = fatty acid, LOOL, and LOH = lipid oxidation products.

forms of iron, ferric (Fe^{2+}) and ferrous (Fe^{3+}), are active in the LOOH decomposition. The rate of Fe^{3+} reduction upon reaction with LOOH has been shown to be much lower than the rate of Fe^{2+} oxidation upon reaction with LOOH [8–10]. However, strong reductants, such as ascorbate and glutathione, can reduce Fe^{3+} at relatively high rates back to the catalytically strong Fe^{2+} [11]. Compounds with chelating properties, such as ethylenediaminetetraacetic acid (EDTA), phosphates, and citric acid, on the other hand, form thermodynamically stable complexes with iron and block the redox cycling activity by occupying all the coordination sites of iron [8].

Lipids are usually dispersed in a complex food matrix, which is often in liquid or semi-liquid state, forming emulsions. Transition metals, iron in particular, are believed to be the major prooxidants in oil-in-water emulsions [12]. Emulsifiers creating a physical barrier around the oil droplets, such as milk and whey protein isolates, may provide adequate protection against iron-mediated oxidation. It is of great advantage when these emulsifiers have also chelating abilities, such as caseinates, transferrin, and lactoferrin. However, some properties of protein emulsifiers may limit their applications. For example, they precipitate close to the isoelectric point and at high ionic strengths, or may increase the viscosity of the emulsion [13]. Phospholipids (PL), on the other hand, are relatively small amphiphilic molecules tolerating environmental changes. The use of marine phospholipids may in addition increase the nutritional value of the products due to the content of LC PUFA. The downside of phospholipids is their zwitterionic character. They may electrostatically attract positively charged iron atoms [14, 15]. This is a problem especially for marine phospholipids, which are prone to oxidation. Food emulsions typically contain a variety of substances dissolved in the aqueous phase, such as salts, proteins/peptides, and carbohydrates. The influence of these components on iron-mediated lipid oxidation in emulsions has rarely been studied.

For a successful addition of marine lipids into food, it is important to understand the pro-oxidation mechanisms of LMW iron as well as how various physicochemical factors and chemical composition affect the pro-oxidative activity. The knowledge may help to develop effective hurdles for minimizing the pro-oxidant activity of ubiquitous LMW iron.

In this work, LMW iron-mediated oxidation in multiphase model systems – liposomes and emulsions – has been studied. Tween 20 and phospholipids of marine and non-marine origin have been used as emulsifiers in cod and herring oil emulsions and for preparation of liposomes. These model systems enable variability and control over multiple parameters – the focus in this study was on iron concentration, pH of the aqueous phase, emulsifier concentration, droplet size and surface charge, lipid characteristics and added salt (sodium chloride) and thickener (xanthan gum). The impact of these factors is discussed in relation to the pro-oxidant activity of LMW iron; mechanistic aspects of

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these interactions are discussed as well. The rate of oxygen disappearance from the systems directly relates to the rate of lipid oxidation, since oxygen is the main substrate for lipid peroxidation. Therefore, measurement of dissolved oxygen in the emulsions/liposomes was used as the principal method to study the LMW iron-mediated oxidation.

2 Materials and methods

2.1 Materials

Herring oil was produced by SINTEF Mobile processing plant in January 2012 from ultra fresh rest raw material from Norwegian spring spawning herring (*Clupea harengus*) obtained from Grøntvedt Pelagic (Uthaug, Norway). The crude herring oil was kept frozen at −30°C until further processing (described in Section 2.3).

Refined cod (*Gadus morhua*) liver oil without added antioxidants was donated by Maritex AS (Sortland, Norway). After opening the bottle, the oil was kept frozen at −20°C under nitrogen.

Mature roe was used for extraction of marine phospholipids. The roe originated from Norwegian spring spawning herring (*Clupea harengus*) and Atlantic cod (*Gadus morhua*). Two types of each roe were obtained: roe within intact roe glands (raw roe) and separated roe grains washed with sea water (washed roe). Herring roe was obtained from Grøntvedt Pelagic (Uthaug, Norway); cod roe was obtained from Hopen Fisk AS (Kabelvåg, Norway). Before extraction (described in Section 2.4) the material was kept frozen at −40°C.

Bacterial phospholipids were isolated from a dried culture of *Methylococcus capsulatus* obtained from Statoil AS – Tjeldbergodden industrial complex (Nordmøre, Norway). Soy phospholipids (Type II-S from soy bean) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2 Chemicals

Tween 20, 2-(*N*-morpholino)ethanesulfonic acid (MES), triphenylphosphine (TPP), butylated hydroxytoluene (BHT), 2-thiobarbituric acid, sodium dithionite ($Na_2S_2O_4$), sodium chloride (NaCl), 1,1,3,3-tetraethoxypropane, 0.1 mol sodium thiosulfate ($Na_2S_2O_3$) aqueous solution, potassium iodide, potassium iodate, sodium sulfite, trichloroacetic acid, *p*-anisidine, boron trifluoride (BF_3), iron (Fe^{3+}) standard (Titrisol), EDTA, xanthan gum, and standards of individual lipid classes were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid (HCl), potassium chloride, ammonium thiocyanate (NH_4SCN), ferrous sulfate ($FeSO_4 \cdot 7H_2O$) and all solvents were supplied by Merck KGaA (Darmstadt, Germany). Anhydrous ferric chloride ($FeCl_3$) was purchased at Riedel de Haën (Seelze, Germany). Sodium hydroxide (NaOH) and ferrous chloride ($FeCl_2 \cdot 4H_2O$) were obtained from Fluka Chemie (Buchs, Germany). Nitrogen (99.99% N_2), hydrogen (99.99% H_2) and helium gas (99.99% He) were provided by AGA AS, Oslo. Standards of FAMEs and lipid classes standard mixes were purchased at Nu-Check Prep Inc. (Elysian, MN, USA). Phospholipid standards were purchased at Avanti Polar Lipids Inc. (Alabama, USA). All chemicals and solvents were of analytical grade, except for solvents used in TLC–FID and GC–FID analyses, which were of chromatography grade. Distilled water was used for preparing aqueous solutions.

2.3 Polishing of crude herring oil

To remove impurities in the crude herring oil, 10% w/w of boiling water (in relation to the oil mass) was added to the crude oil and the mixture was stirred manually by a glass rod for 10 min. The warm mixture was then centrifuged (7000 rpm for 10 min at 40°C), and the clear oil phase was collected, divided into 25 mL portions, and kept frozen at −20°C until needed [16].

2.4 Isolation of phospholipids

The frozen roe was allowed to thaw overnight at 4°C. The extraction of total lipids from the roe was performed according to the method of Bligh and Dyer [17]. The phospholipids (PL) were isolated from the total lipids by precipitation in cold acetone, as described by Kates [18] and modified by Mozuraityte et al. [19]. The precipitation step was performed twice to increase the purity of the isolated PL. The final precipitate was dissolved in chloroform and stored at −20°C until needed. Extraction of PL from the dried bacterial culture followed the same procedure.

2.5 Peroxide value

Pre-formed lipid hydroperoxides (LOOH) were quantified by determination of peroxide value (PV). For the crude and polished herring oil and the soy phospholipids, the iodometric titration method described in AOCS official methods (Cd 8b-90) applied in a titration application issued by Radiometer Analytical SAS [20] was used. The titration end point was assessed potentiometrically using an automatic titrator (TitraLab980) coupled with a single platinum electrode (M21Pt) and a reference electrode (REF 921) (all equipment produced by Radiometer Analytical ASA, Copenhagen, Denmark). The minimum and maximum speed of a standardized titrant (0.01 M $Na_2S_2O_3$) [21] addition was changed to 0.20 and 3.0 mL/min, respectively; the smoothing parameter was set to 1. The PV calculation is described in Eq. (1). The analysis was performed with three parallels, and the results are expressed in mmol LOOH/kg oil as a mean value ± SD. The maximum coefficient of variation (CV) for this method was found 9.1% and the limit of quantification

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(LOQ) was found 1.1 mmol LOOH/kg.

$$PV(mmol/kg) = \frac{C \times (V_s - V_b) \times 2}{G} \times 1000 \quad (1)$$

C is the titrant concentration (average value, $n = 3$) (mol/L), V_s is the titrant consumption for sample (mL), V_b is the titrant consumption for blank (mL), G is the mass of lipids (g), 1000 is a conversion factor for units, and 2 is the stoichiometric molar ratio between LOOH and $Na_2S_2O_3$ [22].

Due to limited quantities, the PV in all the phospholipids and the cod liver oil was determined by the spectrophotometric ferro-thiocyanate assay initially described by Stine et al. [23] and modified as described bellow.

A 100 μL aliquot of PL dissolved in 5-methylpentane was added to a mixture consisting of 5 mL of 96% ethanol and 200 μL of 4% ethanolic BHT solution. Afterwards, 200 μL of a reagent solution prepared by mixing equal volumes of 0.4 M ethanolic NH_4SCN and 4.5 mM $FeSO_4 \cdot 7H_2O$ in 2 M HCl was added. All solutions were deaerated by N_2 gas. The absorbance was read at 500 nm against ethanol exactly 10 min after addition of the reagent solution and the PV was quantified using a linear standard curve prepared with Fe^{3+} standard (0–10 μg). The spectrophotometric mixtures and the reagent solution were kept on ice during analysis. The analysis was performed with 3–12 parallels and the results are expressed in mmol LOOH/kg PL as a mean value ± SE. The calculation of PV is described in Eq. (2).

$$PV(mmol/kg) = \frac{(Abs - Abs_{bl}) \times V}{S \times 55.845 \times 100 \times G} \times 1000 \quad (2)$$

Abs is the absorbance of the sample, Abs_{bl} is the absorbance of the blank (average value, $n = 3$), V is the total volume of 5-methylpentane in which the lipids were dissolved (mL), S is the slope of the standard curve (1/mg), G is the mass of lipids dissolved in 5-methylpentane (g), 55.845 is the molar weight of iron (g/mol), 100 is the aliquot of 5-methylpentane-lipid solution (μL), 1000 is a conversion factor for units.

2.6 *p*-Anisidine value

p-Anisidine value (AV) in the lipids was determined according to the AOCS Official Method [24] using an Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The analysis was performed with three parallels and the results are expressed in AV units as a mean value ± SD.

2.7 Thiobarbituric acid reactive substances

The concentrations of thiobarbituric acid reactive substances (TBARS) in the lipids were determined by a spectrophotometric method described by Ke and Woyewoda [25]. All amounts were reduced to one half relative to the given procedure. The analysis was performed with three parallels for oils and five parallels for PL and the results are expressed in mmol TBARS/kg lipids as a mean value ± SD.

2.8 Total carotenoids

The content of carotenoids in the lipids was determined spectrophotometrically [26]. A portion of lipids was dissolved in *n*-hexane and the absorbance was measured at 472 nm against pure solvent with an Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The carotenoid content was calculated using an absorption coefficient $E = 2100$ L/mol cm, which is the standard absorbance of 1% v/w astaxanthin in *n*-hexane measured at 470 nm. Each sample was analyzed in triplicate and the results are expressed in mg carotenoids/kg lipids as a mean value ± SD.

2.9 Chloride anion content

The content of chloride anions (Cl^-) in the roes and liposomes was measured by titration of halides according to a titration application issued by Radiometer Analytical SAS [27]. The titration end point was assessed potentiometrically using an automatic titrator (TitraLab 980) coupled with a silver electrode (M295Ag) and a reference electrode (REF 921) (all equipment from Radiometer Analytical ASA, Copenhagen, Denmark). The sensitivity and accuracy of the measurements were verified against a standard 0.1 M NaCl solution. The results are given as a mean value ± SD of 3–12 parallels and expressed in μmol Cl^-/g wet roe and μmol Cl^-/g PL for roes and liposomes, respectively.

2.10 Lipid classes

Lipid classes were analyzed by TLC with FID (Iatroscan TLC–FID analyzer MK-6, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Briefly, lipids dissolved in chloroform (10 mg/mL) were injected (3 μL) on silica coated quartz rods (Chromarod-SIII, Mitsubishi Chemical Medience, Tokyo, Japan). The rods were placed into a closed tank containing saturated NaCl solution for 8 min, and afterwards into a development tank containing *n*-hexane/diethyl ether/formic acid (85:15:0.04, v/v/v) for 27 min. The solvent was evaporated from the rods and the rods were scanned. Lipid classes were identified by comparison to the retention times of commercial standards run at the same conditions. Three to five parallels were analyzed for each sample and the results are expressed in % of total peak area as a mean value with maximum CV 4.9%; peak areas $<0.8\%$ were uncertain.

2.11 Fatty acid profile

Methylation of the fatty acids was preformed prior to analysis: 10 mg of lipids in a stoppered glass centrifuge tube were

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dissolved in chloroform containing 10% (in relation to mass of lipids) of internal standard (21:0 fatty acid). The chloroform was completely evaporated by N_2 gas and the lipids were redissolved in 1 mL of 0.5 M NaOH in methanol. The lipids were hydrolyzed for 15 min at 100°C, and cooled. Two milliliter of 10% BF_3 in methanol was added and the mixture was boiled for 5 min at 100°C, and cooled. Afterwards, 1 mL of hexane was added and the mixture was incubated for 1 min at 100°C, and cooled. Finally, 0.5 mL of hexane and 2 mL of saturated NaCl solution was added, the mixture was vortexed and centrifuged at 2000 rpm for 3 min. The hexane phase containing FAMEs was collected, diluted with 0.5 mL hexane, and centrifuged again. The latter step was repeated once more with the collected hexane phase.

The fatty acids in the methylated samples were analyzed according to Dauksas et al. [28] with the following modifications: Agilent Technologies 7890A gas chromatograph with FID (GC–FID) equipped with 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA) was used. The detector temperature was held at 270°C, and the flame was maintained with 25 mL/min H_2 gas and 400 mL/min filtered air. Chromatography was carried out using a Cp-wax 52CB, 25 m × 0.25 mm with id = 0.2 μm column (part no. CP7713, Agilent Technologies). Helium was used as the carrier gas at a flow rate 1.5 mL/min. GC inlets were held at 250°C. The initial oven temperature was held at 80°C and increased to 180°C at 25°C/min, followed by a 2 min hold, after which the temperature was increased to 205°C at 2.5°C/min, followed by a 6 min hold, after which the temperature was increased to 215°C at 2.5°C/min, followed by a 4 min final hold. Fatty acids were characterized by comparison to the retention times of commercial standards and quantified by internal standard. The accuracy of the method was verified by comparison of FA profiles of selected marine oils against profiles assessed by accredited laboratories. Each sample was analyzed in duplicate and % of total peak area was calculated for each fatty acid; peak areas <0.23% were uncertain. The results are expressed in % of total peak area as a mean value with maximum CV 9.6%.

2.12 Preparation of emulsions

All emulsions were oil-in-water type of emulsions. A comprehensive overview on the types of emulsions and liposomes used in the oxygraphic experiments is given in Table 1.

Ten percent w/v herring oil emulsions stabilized with PL were prepared with polished herring oil pre-mixed with PL, and distilled water. The PL originated from either raw herring roe or soy (9%, w/w lipid base). Emulsification was performed with an Ultra Turrax T10 Basic Disperser with 10 mm (diameter) blade (Janke & Kunkel, IKA, Staufen, Germany) applying a gradual increase in the emulsification speed from 8000 to 30 000 rpm. Water phase was slowly poured into the oil during first 5 s of homogenization. Emulsification times of 15 and 30 s, and 1, 2, 3, 4, and 8 min were tested with regard

Table 1. Overview on the liposome and emulsion systems

System	% Total lipids (w/v)	Interface (liposomes) Emulsifier (emulsions), concentration	Oil	Aqueous phase	pH	Iron concentration (M)	Other compounds/conditions
Liposomes	0.6%	raw cod roe PL	–	MES solution	2.0–7.0	10	37°C
						15	–
	0.9%	bacterial PL	–	MES solution	5.5	15, 30, 45	–
	1.5%	raw cod roe PL	–	MES solution	5.5	5, 10, 15, 20, 25, 30	–
						10	200 M TPP in lipid phase
	1.5%	washed cod roe PL	–	MES solution	5.5	5, 10, 15, 20, 25, 30	–
	1.5%	raw herring roe PL	–	MES solution	5.5	5, 10, 15, 20, 25, 30	–
	1.5%	washed herring roe PL	–	MES solution	5.5	5, 10, 15, 20, 25, 30	–
Emulsions	10.0%	raw herring roe PL, 9% (w/w, lipid base)	herring oil	distilled water	3.5–4.0	5, 10, 15, 20, 25, 30	–
						15, 30	0.2% xanthan gum in aqueous phase
	10.0%	soy PL, 9% (w/w, lipid base)	herring oil	distilled water	5.1–5.7	5, 10, 15, 20, 25, 30	–
	5.0%	raw herring roe PL, 9% (w/w, lipid base)	herring oil	distilled water	2.5–7.0	20	–
	0.6%	bacterial PL, 3% (w/w, lipid base)	cod liver oil	MES solution	3.0–7.0	15	–
	0.6%	bacterial PL, 0.5–10.0% (w/w, lipid base)	cod liver oil	MES solution	5.5	20	–
	0.5%	bacterial PL, 3% (w/w, lipid base)	cod liver oil	MES solution	5.5	7.5	0.0–0.1 M NaCl in aqueous phase
	1.5%	Tween 20, 5% (w/w, lipid base)	cod liver oil	MES solution	5.5	15, 400	–
						15	200 M TPP in lipid phase
	1.5%	Tween 20, 2.5–15. % (w/w, lipid base)	cod liver oil	MES solution	5.5	400	–
	0.6%	soy PL, 1.25–15.0% (w/w, lipid base)	cod liver oil	MES solution	5.5	400	–

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to the droplet size distribution (discussed in Section 3.2). Emulsification time of 30 s, which gave the narrowest droplet size distribution, was chosen. For emulsions containing xanthan gum in the mobile phase, 0.2% w/v aqueous solutions of xanthan gum was used instead of distilled water. Emulsification time of 4 min was used due to a higher viscosity of the aqueous phase.

Five percent w/v herring oil emulsions stabilized with herring roe PL were prepared by dilution of the 10% w/v emulsion (described above). Diluted HCl or NaOH (0.01–0.1 M) solutions were used to obtain the desired pH.

0.5% and 0.6% w/v cod liver emulsions stabilized with bacterial or soy PL were prepared by dilution of a 3.0% w/v emulsion prepared with cod liver oil containing 3% PL (w/w lipid base), and 5 mM MES solution, pH 5.5. Emulsification was performed by probe sonication using a 12 mm (diameter) sonication rod (Sonics & Materials Inc., Newtown, CT, USA) under the following conditions: pulse: 6 s repeated 15× (net sonication time: 1.5 min), amplitude: 40%. 5 mM MES solution with pH 3–7 was used for the dilution to obtain the desired pH. For experiments with various % of soy or bacterial PL in the emulsions, the oil contained 0.5–15.0% PL (w/w). For emulsions with 0.0–0.1 M NaCl in the aqueous phase, aliquots of 1.7 M NaCl combined with MES solution were used for dilution of the liposomes to obtain the desired NaCl concentrations.

The PL–oil mixtures were prepared by mixing an aliquot of the oil with an aliquot of phospholipids dissolved in chloroform to a desired concentration. Chloroform was evaporated from the mixtures by a vacuum rotavapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) (1 h, 30°C, 30 mbar) and the mixtures were kept frozen at −20°C until needed.

1.5% w/v cod liver oil emulsions stabilized by Tween 20 were prepared by dilution of 3% w/v emulsion prepared with cod liver oil containing 5% (w/w lipid base) Tween 20, and 5 mM MES solution, pH 5.5. Emulsification was performed by probe sonication using a 12 mm (diameter) sonication rod (Sonics & Materials Inc., Newtown, CT, USA) under the following conditions: pulse: 6 s repeated 30× (net sonication time: 3.0 min), amplitude: 40%. 5 mM MES solution, pH 5.5 was used for the dilution. For experiments with various % of Tween 20 in the emulsions, the oil contained 2.5–15.0% Tween 20 (w/w).

For emulsions with depleted lipid hydroperoxides, an aliquot of 0.5 M TPP in chloroform was added to the oil–Tween mixture to obtain concentration 200 μM TPP in the lipid phase. Chloroform was evaporated from the mixture by a vacuum rotavapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) (1 h, 30°C, 30 mbar).

2.13 Preparation of liposomes

Liposome dispersion was prepared fresh before each set of experiments, as described by Mozuraityte et al. [19]. Briefly, a chloroform solution of PL was evaporated to dryness with a stream of nitrogen gas and the residual solvent was completely evaporated under vacuum (2 h). The dried mass of PL was dissolved in a 5 mM MES solution pH 5.5, to a concentration of 30 mg/mL (3.0%, w/v), and the solution (typically 15–20 mL) was sonicated in a test tube (diameter 2.5 cm) with a Vibra Cell system (Sonics & Materials Inc., Newton, CT, USA) under the following conditions: pulse: 6 s repeated 25× (net sonication time: 2.5 min), amplitude: 50%. The solution was kept on ice both during and after the sonication to minimize oxidation of the lipids. 1.5 and 0.6% liposomes were prepared by dilution of the 3% liposomes with 5 mM MES solution with pH 2–7 to obtain the desired pH.

For liposomes with depleted lipid hydroperoxides, an aliquot of triphenylphosphine (TPP) dissolved in chloroform was added to the phospholipids prior to evaporation of chloroform to obtain 200 μM TPP in the PL phase. For liposomes with 0.1 M NaCl in the aqueous phase, 5 mM MES solution, pH 5.5, containing 0.1 M NaCl was used for sonication of the phospholipids.

2.14 Droplet size distribution

The droplet sizes in 10% herring oil emulsions were measured with a Mastersizer 3000 (Malvern Instruments Ltd., UK). The measurements were performed by adding a few drops of emulsion to a circulating water bath until an obscuration of 6–12% was reached. The emulsions were gently shaken before sampling to eliminate the influence of creaming and flocculation. The refractive indices of cod liver oil (1.481) and water (1.330) were used for particle and dispersant index, respectively. Five parallels were measured and the volume based average droplet diameter (ADD; Dv50) is expressed in μm as a mean value ± SE.

To verify that the droplet size distribution did not change significantly during the time it took to carry out oxidation experiments, the droplet size distribution obtained shortly after the emulsion was made was compared to the distribution determined 4 h after the preparation of the emulsion. The measurements revealed only minimal changes in the droplet size distribution (data not shown), which were neglected.

2.15 Zeta potential and average droplet diameter

The zeta potential measurements in 10% herring oil emulsion and liposomes were performed with a Zetasizer Nano ZS (Malvern Instrument Ltd., UK). The analyses were performed at 25°C. Samples were prepared in two or three parallels, each parallel was measured three times, and the results were pooled. The results are expressed in mV as a mean value ± SD.

The ADD in 0.6% cod liver oil emulsion stabilized with bacterial PL was measured using the same equipment. The

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measurements were performed at 30°C with liposomes diluted to 0.1% with MES solution, pH 5.5. Samples were prepared in three replicates and the results are expressed in nm as a mean value.

2.16 Preparation of iron solutions

Stock solutions of Fe^{2+} and Fe^{3+} (20 mM) were prepared by dissolution of $FeSO_4 \cdot 7H_2O$ and $FeCl_3$, respectively, in 0.5 M HCl to minimize precipitation of iron. Working solutions of Fe^{2+} and Fe^{3+} were prepared daily by diluting an aliquot of the stock solutions with distilled water to a desired concentration. For addition of different concentrations of iron into the emulsions and liposomes, the volume of the dose (μL) was kept constant while the concentration of the working solution was varied.

2.17 Oxygen uptake measurement

Oxidation of fatty acids in emulsions/liposomes was followed by measuring dissolved oxygen consumption using an Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK) as described in earlier studies [10, 19, 29, 30]. Briefly, 1 mL of liposomes/emulsion was transferred into an oxygraphic cell surrounded by a water jacket to maintain a constant temperature. The concentration of the dissolved oxygen was measured by a Clark polarographic oxygen electrode placed inside the cell. The electrode was calibrated with oxygen saturated and oxygen depleted distilled water; oxygen was depleted by adding $Na_2S_2O_4$. The cell was equipped with a magnetic stirrer and closed with a plunger with a capillary opening preventing access of air oxygen and at the same time allowing injection of solutions. The concentration of the dissolved oxygen (μM) was continuously recorded as a function of time (min), giving continuous oxygen concentration curves. In order to re-establish saturation conditions, infusion of oxygen was performed when the concentration of the dissolved oxygen in the cell reached almost zero. As a measure of oxidation, oxygen uptake rates (OUR) were calculated from the recorded curves. Three cells were run simultaneously for each experiment and the OUR were expressed in μM O_2/min as a mean value ± SD.

2.18 Oxidation experiments

The majority of the oxidation experiments were performed at 30°C and at ambient light conditions. Some experiments were performed at 35 and 37°C (indicated in the text). The pH of liposomes/emulsions was measured after the preparation (pH of the batch) and again after each oxidation experiment (pH for the experiment), because addition of acidic iron solutions slightly lowered the initial pH.

A representative curve of oxygen consumption in emulsion/liposomes and the principle of OUR calculations are shown in Fig. 2. Linear background oxygen consumption was recorded for 5–10 min before addition of a prooxidant, a background OUR was measured at this stage (r_0). The prooxidant was added to initiate oxidation and the oxygen concentration was followed for 10–30 min depending on the progress of the oxidation, total OUR was measured at this stage (r_1). The net OUR was found by subtracting the total OUR from the background OUR ($r = r_1 - r_0$). In experiments with Fe^{2+}, the magnitude Δ (μM) of the drop in the oxygen concentration, which occurs immediately after addition of Fe^{2+}, was quantified as well. This drop is referred to as "the initial drop" in the text (discussed in Section 3.4). The recordings of oxygen uptake curves, quantification of OURs and determination of oxygen concentrations at any time point on the curve were done by means of Oxyg32 software. Specific OUR was calculated as net OUR divided by Fe^{2+} concentration (M O_2/M Fe^{2+}/min).

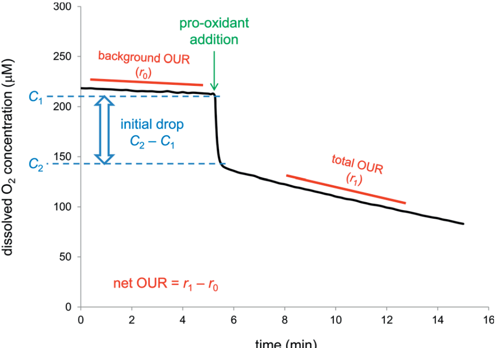

Figure 2. A representative oxygen concentration curve for emulsion/liposomes with addition of a prooxidant (Fe^{2+}). OUR measurement and quantification of the initial drop in oxygen concentration after addition of Fe^{2+} is indicated in the figure.

2.19 pH verification

The pH of solutions, liposomes and emulsions was measured by a TIM900 Titrator manager (TitraLab, Radiometer Analytical, Copenhagen, Denmark) coupled with a combination glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, USA), which was calibrated daily against standard buffer solutions, pH 4.0 and 7.0, at 22°C.

2.20 Statistical analysis

Microsoft Excel 2010 was used for calculations and data processing. A statistical program Minitab (version 16.2.3) was used for statistical analyses. To assess significant differences, the data were subjected to analysis of variance (one-way ANOVA), followed by a Tuckey test.

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3 Results and discussion

3.1 Characterization of lipids

The crude oil from herring was expected to contain impurities commonly present in crude oils, such as proteins, free fatty acids, phospholipids, minerals, antioxidants, pigments, sterols, and insoluble particles [31]. To remove these impurities, the oil was polished by hot water. The impact of the polishing step on the oil characteristics – oxidation status, fatty acid profile and the content of carotenoids – was measured (Table 2).

The values for PV, AV, and TBARS were slightly higher for the polished oil than for the crude oil indicating that minor oxidation of the lipids had taken place during the polishing treatment. There was a significant decrease ($p < 0.05$) in the carotenoid content (56%), clearly showing that a proportion of carotenoids was removed or depleted by the polishing step. The polishing step improved clarity of the oil (not shown), but did not have any effect on the lipid classes composition (Table 2), which indicates that mainly non-lipid material was removed.

The oxidation status of the different PL is given in Table 2. PV in the herring roe PL were more than two-fold higher compared to PV in the cod roe PL, and also AV and TBARS were higher for the herring roe PL than for the cod roe PL. The lowest PV values were found in soy and bacterial PL. The purity of all the phospholipids was found to be >99.5%.

Fatty acid (FA) profiles revealed large differences in the proportions of mono-, di- and poly-unsaturated fatty acids (double bond >3, C ≥18) and the levels of EPA and DHA in the different lipids (Table 2). The content of PUFA decreased in order: herring roe PL>cod roe PL>cod liver oil>herring oil>soy PL>>bacterial PL.

3.2 Preparation and characterization of herring oil emulsions

Herring oil emulsions (10%, w/w) stabilized by herring PL were prepared by homogenization of an oil-PL mixture with distilled water. It was desirable to obtain emulsions with small droplets and narrow droplet size distributions, which would not change their physicochemical properties over at least a 4 h period required for conducting a set of oxygraphic experiments. Emulsions with small droplets are almost always more stable against creaming, coalescence and often also flocculation [32] and a narrow size distribution is important from the oxidation point of view, since variation in the size of the droplets, i.e. total surface area, may influence the rate of lipid oxidation [33]. The effect of droplet size on oxidation rates is discussed in Section 3.6. Emulsification time is one of the parameters influencing the droplet size distribution.

Emulsification times ranging from 15 s to 8 min were tested. A shorter emulsification time (≤2 min) resulted in smaller droplets and a narrower droplet size distribution, except for the shortest emulsification time (15 s), which gave larger droplets than 30 s (Fig. 3). This could be due to insufficient energy supply to break up the droplets [32]. Longer emulsification times (>2 min) gave asymmetrical distributions and larger droplets occurred in the distribution. The longer homogenization may support coalescence of droplets, which could explain the latter phenomenon. Independent of the emulsification time, all the emulsions had a tendency to cream shortly after preparation.

During the oxidation experiments employing the oxygraphic system, the size of the droplets in emulsions might influence the rate of lipid oxidation, but creaming would not be of great importance as the emulsions are continuously and evenly stirred inside the oxygraphic cells. Therefore the emulsification time of 30 s giving the narrowest droplet size distribution was chosen for preparation of herring oil emulsions.

Average droplet size and size distribution curves of these emulsions prepared at five different occasions differed marginally (data not shown). The pooled Dv $50 = 10.9 \pm 0.1$ (SE) μm ($n = 25$). Zeta potential, which reflects the surface charge of the particles, was -13 ± 3 (SD) mV ($n = 5$) at pH 5.5 ± 0.2.

3.3 Characterization of liposomes

Marine liposomes were prepared from phospholipids isolated from raw roes and roes that were separated and washed with seawater. Measurements of chloride anions (Cl^-) revealed that the separated washed roes contained significantly higher levels of Cl^- than the raw roes and the same situation was found for liposomes prepared from the corresponding PL (Table 3).

It is clear that the extraction and isolation techniques did not eliminate Cl^- from the PL fraction. The data indicate that Cl^- (and possibly also the counter ions, such as Na^+, K^+) were strongly associated with the phospholipids and withstood the isolation conditions. Phospholipid heads are zwitterions bearing both positive and negative charge, and may electrostatically bind both cations and anions [14, 15], which could explain the presence of chlorides.

At pH 5.5, the zeta potential of the different liposomes (1.5%) was found to be negative but with varying values (Table 3). The highest zeta potential was found for liposomes containing the highest concentration of Cl^-. The impact of the inherent Cl^- as well as exogenously added Cl^- as NaCl on iron-mediated lipid oxidation is discussed in Section 3.9.

3.4 Iron-mediated lipid oxidation in liposomes and emulsions

LMW iron-mediated lipid oxidation in different emulsions and liposomes containing marine lipids (overview in Table 1) was followed by recording dissolved oxygen consumption over time, as shown in Fig. 4A–D. Oxygen consumption curves for both the emulsions and liposomes were

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Table 2. Oxidation status characterized by PV, AV and TBARS, carotenoid content and composition of the different phospholipids and oils

	Phospholipids originating from						Herring oil		Cod liver oil
	Herring raw roe	Herring washed roe	Cod raw roe	Cod washed roe	Soy	Bacteria	Crude	Polished	Refined
PV (mmol LOOH/kg)	37.6 ± 1.1	35 ± 2	15.2 ± 1.1	9.7 ± 0.8	8.1 ± 1.4	1.6 ± 0.2	6.31 ± 0.04	7.47 ± 0.09	7.3 ± 0.4
AV	16.5 ± 0.8	20.3 ± 0.6	NA	16.4 ± 1.2	NA	NA	1.15 ± 0.15	1.61 ± 0.12	NA
TBARS (mmol/kg)	1.1 ± 0.3	1.0 ± 0.1	NA	0.47 ± 0.5	NA	NA	0.40 ± 0.04	0.47 ± 0.03	NA
Total carotenoids (mg/kg)	10.8 ± 0.7	9.1 ± 0.8	7.9 ± 0.7	14.7 ± 1.1	NA	NA	3.2 ± 0.6	1.80 ± 0.09	NA
Lipid classes (%)									
TAG	<LOD	<LOD	<LOD	<0.8	<LOD	<LOD	99.2	98.9	NA
Cholesterol	<0.8	<0.8	<0.8	<0.8	<LOD	<LOD	<0.8	<0.8	
Unspecified	<0.8	<0.8	1.1	<0.8	<0.8	<0.8	<0.8	<0.8	
PL	99.5	99.7	98.2	98.3	99.5	99.5	<0.8	<0.8	
Fatty acid profile (%)									
Saturated	27.8	29.2	33.3	26.6	25.9	50.1	NA	22.4	18.0
Mono-unsat. of which	16.7	16.9	24.6	23.1	8.7	46.9[a]		59.1	51.1
CET	2.9	3.5	0.5	2.2	9.2	<0.2		38.1	16.1
ERU	0.5	0.6	2.3	<0.2	1.4	<0.2		2.2	<0.2
GAD	9.2	11.5	17.1	18.6	1.3	<0.2		25.8	24.0
OLE	35.2	33.7	44.9	55.4	76.2	0.8		19.7	36.8
di-unsat.	1.0	0.9	1.4	0.7	56.7	<0.2		1.7	1.7
Poly-unsat. (db $\geq$ 3) of which	54.5	53.1	40.6	46.0	8.7	2.9[b]		16.8	29.2
EPA	23.4	24.8	32.5	26.6	1.7	1.1		40.5	30.6
DHA	69.0	65.7	62.3	66.3	2.8	<LOD		40.0	48.4
DPA	2.0	2.9	2.3	1.8	<0.2	<LOD		4.3	3.7

NA, not analyzed; LOD, limit of detection; db, double bond; the values are given as a mean value ± standard error or standard deviation or with a coefficient of variance of *n* measurements (specified in Section 2 under each analysis); CET, cetoleic aid (C22:1 n11), ERU, erucic acid (C22:1 n9), GAD, gadoleic acid (C20:1 n11); OLE, oleic acid (C18:1 n9); EPA, eicosapentaenoic acid (C20:5 n3); DHA, docosahexaenoic acid (C22:6 n3); DPA, docosapentaenoic acid (C22:5 n3); unsat., unsaturated.

[a] The predominant mono-unsaturated fatty acid in bacterial phospholipids is palmitoleic acid (C16:1 n7 *cis*).

[b] The predominant poly-unsaturated fatty acid in bacterial phospholipids is eicosatrienoic acid (C20:3 n3).

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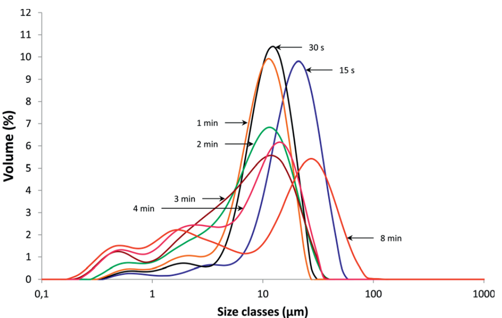

Figure 3. Particle size distributions in 10% (w/w) herring oil emulsions stabilized by raw herring phospholipids prepared with different emulsification times.

characterized by a linear background OUR varying between 0.2 and 5.0 $\mu M\ O_2$/min.

Addition of ferric (Fe^{2+}) ions to both the emulsions and liposomes resulted in a rapid drop in the oxygen concentration immediately after the addition of Fe^{2+} (referred to as "the initial drop"), which was followed by a linear decrease in the oxygen concentration. When ferrous (Fe^{3+}) ions were added, no initial drop but only the linear decrease in oxygen concentration was observed (Fig. 4A).

Mozuraityte et al. [10] explained that the initial drop in LMW iron-mediated oxidation in cod liposomes was due to establishment of a concentration equilibrium between Fe^{2+} and Fe^{3+} via rapid oxidation of Fe^{2+} to Fe^{3+} by pre-formed lipid hydroperoxides (LOOH). It is known that Fe^{2+} decomposes LOOH by a rate an order of magnitude higher than the rate for Fe^{3+} [34, 35]. The rapid conversion of Fe^{2+} into Fe^{3+} generates alkoxyl radicals ($LO^{\bullet}$) which rapidly abstract hydrogen from adjacent fatty acids producing lipid alkyl radicals ($L^{\bullet}$) which than rapidly react with dissolved oxygen, causing the initial drop in dissolved oxygen concentration. The linear decrease in the oxygen concentration takes place once the equilibrium between Fe^{2+} and Fe^{3+} is established and a slower reaction between Fe^{3+} and LOOH becomes rate limiting. As a consequence, the concentration of Fe^{2+} and Fe^{3+} becomes equal regardless of the original ions, and each iron atom oscillates between both forms. The reaction scheme for iron-catalyzed lipid oxidation is depicted in Fig. 1. Since the magnitude of the initial drop can give additional information on iron-mediated oxidation, for example, whether added compounds interact with Fe^{2+}, most experiments in this study were performed using Fe^{2+}.

The oxygraphic curves in Fig. 4A and B clearly show the establishment of the equilibrium between Fe^{2+} and Fe^{3+}, i.e. the initial drop, not only in marine liposomes, but also in emulsions stabilized with either marine PL or Tween 20. This was expected since the mechanism by which LMW iron acts as a pro-oxidant, i.e. red-ox cycling of iron upon decomposition of hydroperoxides, should not be dependent upon the type of lipid system.

The concentration of pre-formed LOOH in the different phospholipids and oils varied (Table 2). From a purely mechanistic point of view, the higher the number of pre-formed LOOH (related primarily to the emulsifier interface), the higher the number of potential locations for initiation of iron-mediated oxidation. Therefore, for emulsifiers with higher PV levels, such as the marine PL, higher rates of oxygen consumption and also larger initial drops could be expected. This would however only be valid if iron were in excess of the pre-formed LOOH (on a molar basis). In all types of liposomes and emulsions the concentrations of pre-formed LOOH in the interfaces were found to be higher (based on PV determined in pure PL; Table 1) than the amount of added iron. Therefore, the concentration of pre-formed LOOH was not the major limiting factor for iron-mediated oxidation. Differences in oxidation rates, the magnitudes of the initial drop and susceptibility to oxidation for liposomes and emulsions in this study should therefore be attributed to other factors than endogenous LOOH content. A number of factors is discussed in the sections below.

The initial drops in the emulsions indicate that both marine PL and Tween molecules were peroxidized to some degree. Pre-formed lipid hydroperoxides in the marine PL

Table 3. Chloride content in different roe types and 1.5% liposomes prepared from PL isolated from the roes, and zeta potential in the liposomes at pH 5.5 and 3.5, and in liposomes containing 0.1 M NaCl

	Roe	1.5% liposomes	1.5% liposomes		1.5% liposomes 0.1 M NaCl
Type or roe	Chloride content (µmol/g wet roe)	Chloride content (µmol/g PL)	Zeta potential (mV) at pH 5.50 ± 0.05	Zeta potential (mV) at pH 3.5 ± 0.1	Zeta potential (mV) at pH 5.50 ± 0.05
Raw herring roe	10.7 ± 0.1	<LOD	−26.2 ± 4.7	−5.3 ± 0.3	−5.7 ± 0.3
Washed herring roe	52.8 ± 1.2	35 ± 2	−50.4 ± 1.7	−28.8 ± 1.5	−12.1 ± 0.3
Raw cod roe	NA	<LOD	−37.2 ± 0.5	−19.1 ± 0.9	−6.0 ± 0.2
Washed cod roe	NA	152 ± 2	−8.1 ± 0.6	+4.6 ± 0.4	0.0 ± 0.1

NA, not analyzed; LOD, limit of detection.

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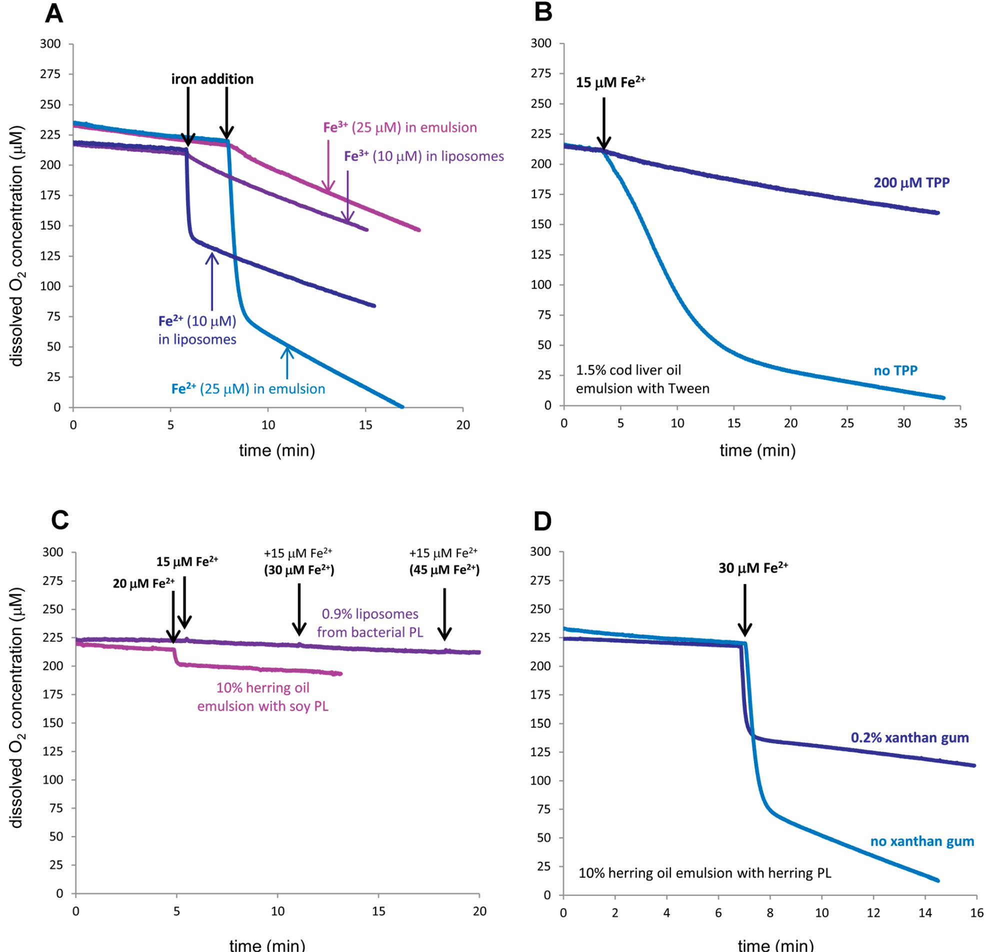

Figure 4. Oxygen consumption curves for different emulsions/liposomes with addition of iron ions (indicated by arrows). (A) Herring oil emulsion stabilized with raw herring roe phospholipids (PL) versus liposomes made from raw herring roe PL; (B) cod liver oil emulsion stabilized with Tween with and without endogenous hydroperoxides depletion by triphenylphosphine (TPP); (C) liposomes made from bacterial PL and herring oil emulsion stabilized with soy PL; (D) 10% herring oil emulsion stabilized with raw herring roe PL with or without xanthan gum dissolved in the aqueous phase.

were indeed determined (Table 2); the PV in Tween 20 was not measured. Nevertheless, a study of Mancuso et al. [36] showed that Tween 20 molecules contain various levels of hydroperoxides, depending on the age of the chemical. These hydroperoxides were decomposed upon contact with Fe^{2+} ions added into Tween 20 micellar dispersions, which agrees very well with the oxygraphic curves recorded in this study.

Alternatively, peroxidized TAGs in the core of the lipid droplets could be responsible for oxidation of Fe^{2+} to Fe^{3+}. However, the net OUR in Tween emulsions did not increase to any larger degree for 15 μM Fe^{2+} nor for 27-fold higher Fe^{2+} concentration (400 μM), nor for different concentrations of Tween (Fig. 7). These experiments indicate that the lipids in the core of the droplets were only marginally involved in iron-mediated oxidation. In other words, after the hydroperoxide groups (–OOH) in Tween molecules were broken, the resulting radicals did not propagate oxidation of fatty acids located in the core of the droplets to any

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significant levels, and most likely reacted with adjacent water molecules. The overall effect could also be interpreted as following: once the hydroperoxides in Tween were broken down, iron-mediated oxidation was prevented. In this view, Tween molecules formed a barrier around the lipid droplet preventing interactions between iron atoms and core lipids. These outcomes are in contrast to a study by Nuchi et al. [37] in which it was proposed that Tween-OOH can stimulate the oxidation of the core lipids in emulsions when LMW iron is added into the aqueous phase as a pro-oxidant.

As shown in Fig. 1, the red-ox cycling of iron is triggered by pre-formed lipid hydroperoxides (LOOH) and at the same time the cycle facilitates formation of new LOOH, which will be further decomposed by iron. Thus, the red-ox cycling is sustained as long as there are enough available oxidizable fatty acids in the system. If pre-formed LOOH are not present, peroxidation should not be initiated after addition of iron. It is possible to chemically break down the majority of the pre-formed lipid LOOH by an excess of triphenylphosphine (TPP), which reduces the hydroperoxide group (–OOH) into an alcohol group (–OH) [10].

In 1.5% cod liver oil emulsion stabilized by Tween, depletion of pre-formed lipid hydroperoxides (both in the emulsifier and the oil) by TPP prevented the rapid oxidation of Fe^{3+} to Fe^{2+} (Fig. 4B). Similarly, addition of Fe^{2+} and Fe^{3+} to peroxide depleted marine liposomes did not increase OURs to any significant levels (data not shown). These oxygraphic experiments show a crucial dependency of the pro-oxidant activity of iron on pre-formed lipid hydroperoxides, and the outcome is in agreement with other studies [11, 38].

In experiments with 0.9% liposomes made from bacterial PL, addition of Fe^{2+} (15–45 μM) did not give the initial drop, and the OUR was only marginally increased with increasing iron concentration (Fig. 4C). The bacterial PL had a low PV level and contained 97% saturated + mono-unsaturated fatty acids (Table 2). Abstraction of a hydrogen atom from a saturated and mono-unsaturated fatty acid by alkoxyl lipid radical is energetically demanding [9], which could explain the low susceptibility of bacterial PL in liposomes to oxidation. The impact of unsaturation level of the lipids on iron-mediated oxidation is discussed in Section 3.7.

On the other hand, in experiments with 0.6% cod liver oil emulsions stabilized by bacterial PL, addition of iron did have a significant pro-oxidant effect (data in Fig. 7). Since the interfacial layer in the emulsions was composed of bacterial PL, which showed a high resistance to iron-mediated oxidation in liposomes, it is reasonable to assume that pre-formed LOOH in the core of the emulsion droplets, i.e. in the cod liver oil, were responsible for the oxidation. The question is how hydroperoxide groups (–OOH) associated with the hydrophobic fatty acid chains in TAG molecules, which are located in the core of the emulsion droplet, come into contact and interact with iron atoms dissolved in the aqueous phase.

In order to facilitate the reaction, the –OOH group has to come in direct contact with the iron atom. The surface potential of the aqueous phase adjacent to the phospholipid heads has been found negative [39]. It has been generally accepted that the interaction between –OOH and iron atoms is aided by an electrostatic attraction between the negative surface charge of the phospholipid interphase and the positive charge on the iron atom [14].

Peitzsch et al. [39] proposed that hydration of the phospholipid heads forms a water shell around the heads, which is impenetrable for ions dissolved in the aqueous phase. On the other hand, other studies suggest that ions can penetrate this water shell and that water molecules are involved in the association (affinity) between the ions and the phospholipid heads [8, 15].

The phosphate group within the phospholipid head (PL-phosphate) has been proposed as the binding site for a metal ion [15]. To retain the pro-oxidant activity, the iron atom needs to be capable of red-ox cycling once associated with the PL-phosphate. This means that at least two co-ordination sites on the iron atom must be unoccupied. Several (possibly four) PL-phosphates could be involved in "trapping" the iron atom in a plane and still allowing the red-ox cycling. Similar type of "complexation" can be seen for example in heme-groups, where the iron atom is immobilized within a porphyrin ring by four bonds while it retains its valence status and pro-oxidant activity [40]. Once iron atoms are associated with the phospholipids, they should be less available for competing reactions, such as precipitation or complexation by chelators. Indirect evidence for the latter two reactions is further discussed in Sections 3.5 and 3.8. It is likely that iron atoms bound to the phosphate groups affect the fluidity of the membrane. Borst et al. found that membrane fluidity was reduced when Fe^{3+} was added to microsomal membranes and the reduction was iron concentration dependent [41].

Due to six double bonds, the carbon chain of DHA molecule can assume hundreds of bent conformations resembling coils [1]. Hydroperoxide groups are preferably formed on C20 atom of DHA chain [9, 42], i.e. near the free end of the chain. The –OOH group has been reported to have a large dipole moment giving the group a hydrophilic character [43]. This might draw the part of the molecule bearing the –OOH group towards the phospholipid head and the aqueous phase. The coiled carbon chain, the C20 location and the polar character of the –OOH group might allow the group to protrude into the interfacial regions and thus come into contact with the iron atom located within the phospholipid heads.

Fukuzawa et al. [11] proposed a site-specific mechanism of lipid peroxidation in PL membranes, where the –OOH group is cleaved by iron near the interface and the resulting lipid radicals then penetrate back into the hydrophobic region propagating oxidation upon generation of new –OOH groups, which then move towards the surface again to be cleaved by iron. Packing of PL molecules, which contain unsaturated

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fatty acids is less tight and the membrane fluidity therefore increases [44]. This may allow the entire TAG to be drawn into the emulsifier interface. Assuming that the peroxidized TAG are dynamically exchanged with un-oxidized TAG deeper in the core of the emulsion droplets, the supply of –OOH to iron atoms can then be facilitated even from the core of the emulsion droplet.

In 10% herring oil emulsions stabilized by soy PL, an increase in oxygen consumption due to addition of Fe^{2+} was not observed (Fig. 4C). This is opposite to the 0.6% cod liver oil emulsions stabilized by bacterial PL, but similar to the cod liver oil emulsions stabilized by Tween. The data suggest that soy PL prevented the pre-formed LOOH in TAG from reaching iron ions and were not readily oxidized themselves, even though soy PL contain PUFA (8.7%) and di-unsaturated FA (76.2%; Table 2).

It should be noted that the characteristics of the fish oils were different, which could have an impact on the overall susceptibility to iron-mediated oxidation. Cod liver oil was refined and contained 29.2% PUFA, while herring oil was crude and purified by polishing, containing only 16.8% PUFA. Crude oils have been known to display better oxidative stability than refined oils due to content of natural antioxidants [45] and the levels of endogenous tocopherols were not quantified in any of the lipids.

The latter examples indicate that interactions between iron and peroxidized fatty acids may be affected by overall composition of lipid droplets, such as the exact type of emulsifier and its concentration, in addition to the type and amount of fish oil. The influence of the emulsifier quantity in emulsions on Fe^{2+}-mediated lipid oxidation is further discussed in Section 3.6.

The relationship between Fe^{2+} concentration (7.5–30.0 μM) and the net OUR and the magnitude of the initial drop was plotted for herring oil emulsions and marine liposomes (Fig. 5). In all the systems, the net OUR was linearly correlated ($p < 0.05$) with Fe^{2+} concentration and a significant linear correlation ($p < 0.05$) was also found between the amount of oxygen consumed within the initial drop and the concentration of Fe^{2+}. The specific OUR calculated from the data in the graphs and the slopes of the linear dependencies between the initial drop and iron concentration for all the systems are given in Table 4. Since the level of pre-formed LOOH is not a limiting factor for iron-mediated oxidation, as explained above, the linear dependencies indicate that the added iron is mainly responsible for mediating the oxidation.

The specific OUR calculated for Fe^{2+}-mediated oxidation (Table 4) show that iron activity significantly differs between the different types of liposomes and emulsions. Nevertheless, the mechanism of iron-mediated oxidation appears to be the same. Increase in iron concentration leads to a proportional increase in oxidation, given the presence of endogenous LOOH to trigger the oxidation and the excess of double bonds to sustain it.

For the herring oil emulsion (1% PL), the initial drop in oxygen concentration was 5.00 ± 0.29 fold larger than the concentration of Fe^{2+}. This means that in this particular system, one Fe^{2+} ion was responsible for consumption of five O_2 molecules within the red-ox cycle. A similar number (4.57 ± 0.25) was found for liposomes prepared from the same phospholipids (1.5%) as those in the emulsion. Other systems showed significantly different numbers (Table 4). The number of O_2 molecules consumed within one red-ox cycle is therefore not constant, but seems dependent on the environmental conditions and the characteristics of the lipids. The effect of pH, Cl^-, NaCl, lipid characteristics, and emulsifier quantity on iron-mediated oxidation will be discussed in the following sections.

3.5 Effect of pH

The acidity (pH) of the aqueous phase plays an important role in oxidation of lipids in multiphase systems because of the impact on the emulsifier charge and solubility of iron [12]. The primary location of the added iron was the aqueous continuous phase. The effect of pH on iron-mediated oxidation was measured in the pH range 2–7 for different liposomes and emulsions; the pH curves are shown in Fig. 6.

The optimal pH for iron-mediated oxidation was found in the pH range 4.5–5.5. This range was consistent for all the systems, which varied in the origin of lipids, lipid concentration, temperature and complexity (i.e. liposomes vs. emulsions).

Mozuraityte et al. [19] found a bell shaped pH-curve for LMW iron-mediated oxidation of cod roe PL in liposomes, with the highest oxidation rates at pH 4–5. The peak was explained by increasing electrostatic attraction between iron ions and liposome droplets with decreasing pH and by impaired solubility of iron at $pH > 5$. Sørensen et al. [46] found that lipids in Tween emulsions oxidized faster at pH 3 than at pH 6. The latter observation was explained by increased solubility of iron at low pH. Emulsions stabilized with anionic emulsifiers have been found to be more oxidatively stable at low pH (0–3) due to neutralization of the negative surface charge of the droplets by hydrogen protons (H^+) which causes weaker attraction or even repulsion of positively charged iron ions. [12] The pH curves in this study are largely in accordance with these studies.

The zeta potential near the surface of the oil droplets in the herring emulsions stabilized by herring PL was found to be -13 ± 3 mV at pH 5.5 ± 0.2. The zeta potential typically becomes less negative or even positive when the pH is decreased. Therefore, it can be expected that the zeta potential in the emulsion became less negative after addition of the acidic iron solution. This trend was observed in liposomes – the zeta potential was less negative or even positive at pH 3.5 compared to pH 5.5 (Table 3). The inhibition of iron-mediated oxidation at low pH (<4.5) is therefore most likely caused by the less negative zeta potential

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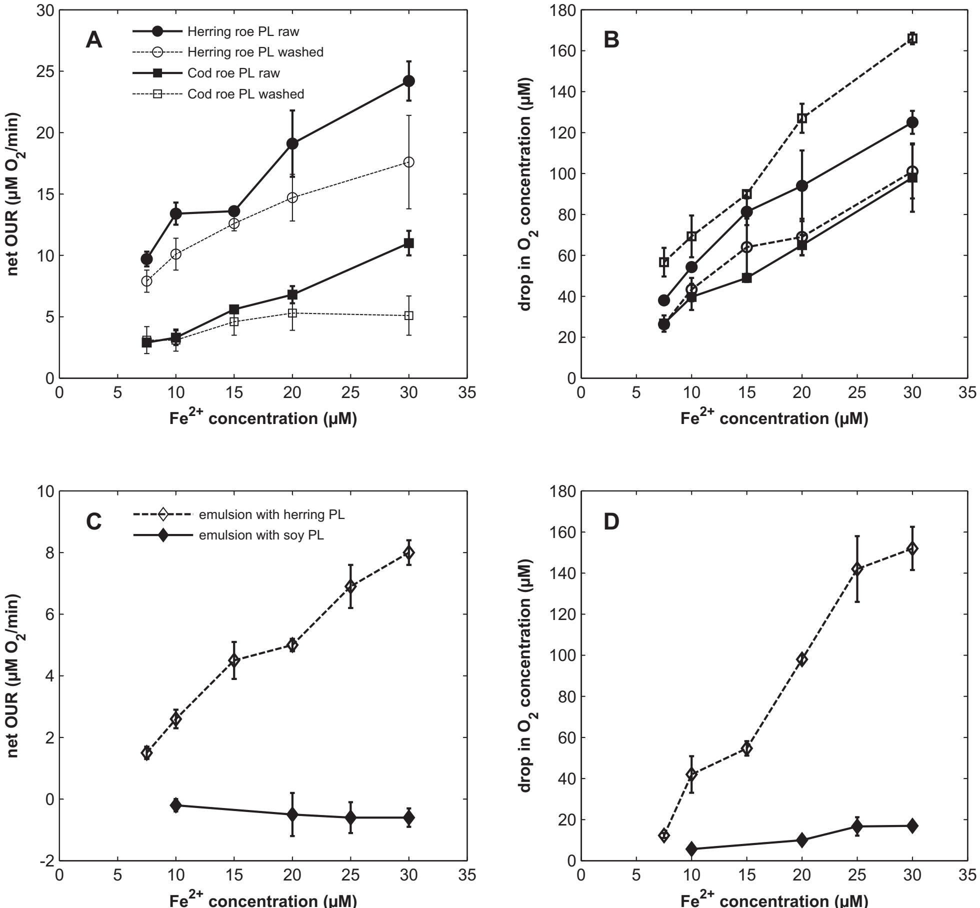

Figure 5. Net OUR and magnitude of the initial drop in Fe^{2+}-mediated oxidation (7.5–30 μM) in 1.5% liposomes made from different marine phospholipids (PL) (A) and (B), and in 10% herring oil emulsions stabilized with herring PL (C) or soy PL (D). The results are mean values of three (except for 25 μM Fe^{2+} in C and D where $n = 15$) parallels ± SD.

of the oil droplets causing weaker electrostatic attraction between iron ions and the oil droplets.

Iron solubility is another parameter governed by pH. Fe^{3+} precipitates as iron(III) hydroxide ($Fe(OH)_3$) at neutral pH, while Fe^{2+} is more tolerable to neutral pH than Fe^{3+}, as seen on solubility product constants (K_{sp} $Fe(OH)_2 = 4.87 \times 10^{-17}$; K_{sp} $Fe(OH)_3 = 2.79 \times 10^{-39}$) [47]. This means that solubility of Fe^{2+} and Fe^{3+} in water (pH = 7) is 2.3 μM and 0.10 nM, respectively. At acidic conditions (pH < 7) the solubility of Fe^{2+} is greatly improved, allowing high concentrations (M) already at pH 5.0; the solubility of Fe^{3+} however remains very low (nM). Upon red-ox cycling of free iron, iron atoms at Fe^{3+} state should therefore largely precipitate, which would slow down the oxidation of fatty acids considerably. This is not observed in the oxygraphic measurements, because iron is more or less active in the whole acidic pH range.

In Section 3.4, it is postulated that iron atoms are bound by the PL-phosphate groups, which could explain the oxidation activity of iron at pH from 5 to 7. However, the proportion of iron, which is bound to PL-phosphates is not known and may vary. The binding constant for iron and PL-phosphates is not known either. The decrease in oxidation at pH > 5.5 could therefore be attributed to a partial precipitation of iron as $Fe(OH)_3$ rather than to interactions between iron and the droplets, as zeta potential is more negative at increasing pH and iron ions should therefore be strongly attracted to the surface of the particles.

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Table 4. Susceptibility to Fe^{2+}-mediated oxidation in different emulsions and liposomes expressed as specific OUR and the slope of the linear dependences between Fe^{2+} concentration and the magnitude of the initial drop

	Specific OUR ($M\ O_2/M\ Fe^{2+}\ min^{-1}$)	Iron conc. versus magnitude of the initial drop Slope ($\Delta M\ O_2/M\ Fe^{2+}\ min^{-1}$)
1.5% liposomes (pH 5.5) with PL from		
Raw herring roe	1.06 ± 0.23	4.57 ± 0.25
Washed herring roe	0.84 ± 0.20	3.57 ± 0.18
Raw cod roe	0.36 ± 0.04	3.31 ± 0.09
Washed cod roe	0.29 ± 0.12	5.94 ± 0.26
10% herring oil emulsion stabilized with		
Raw herring roe PL	0.28 ± 03	5.00 ± 0.29
Soy PL	-0.02 ± 0.02	0.58 ± 0.03

3.6 Impact of emulsifier concentration and particle size

The concentration of emulsifier in emulsions affects the droplet size, total surface area, and the physical stability of the emulsions [32]. Fe^{2+}-mediated oxidation in 0.6% cod liver oil emulsions with different levels of soy PL (1.75–15%, w/w oil basis) or bacterial PL (0.5–10%, w/w oil basis) and Tween 20 (2.5–15%, w/w oil basis) was measured. The relationship between % of emulsifier and the net OUR for Fe^{2+}-mediated oxidation is shown in Fig. 7. The pro-oxidant activity of Fe^{2+} varied for different levels of PL, being highest at PL levels 5–10%. On the other hand, in emulsions stabilized by Tween 20 (2.5–15.0%, w/w oil basis) the amount of emulsifier did not affect the activity of iron.

It should be noted that the concentration of iron in the emulsions stabilized by soy PL and Tween 20 was increased to 400 μM in order to see any effect. At lower iron concentrations, which were effective in all other experiments (10–30 μM), the specific OUR was $<0.01\ M\ O_2/M\ Fe^{2+}\ min^{-1}$, giving inconclusive results.

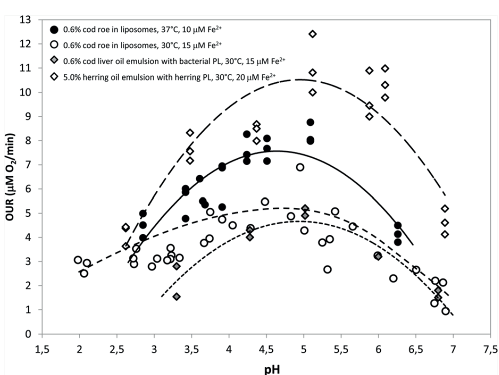

Figure 6. Influence of pH on Fe^{2+}-mediated oxidation in different emulsions and liposomes. Each point represents a single oxygraphic measurement.

Investigation of droplet sizes in the emulsions revealed that higher PL concentrations resulted in formation of smaller droplets (inset in Fig. 7), thus creating a larger total surface area. Some studies have proposed that oxidation rates do not change dramatically with large variations in surface area, since the surface in most cases is large enough not to limit the reaction rates [12]. However, as explained in Section 3.4, the pro-oxidant activity of iron is dependent on the encounters of iron atoms with pre-formed LOOH. The larger the area, the higher the likelihood of these encounters. This could explain the increase in iron activity for higher PL concentrations, since the encounter of the pre-formed LOOH with iron atoms is likely to be higher due to smaller droplets.

The decrease in the activity of iron at PL concentrations >10% could be attributed to micelle formation which may take place once the emulsifier no longer contributes to droplet formation and the PL molecules in the aqueous phase reach the critical micelle concentration [8]. A proportion of iron ions may be associated with the micelles, thus not involved in oxidation of fatty acids in the lipid droplets [48].

Liposomes from raw herring roe PL were much more susceptible to iron-mediated oxidation than emulsions containing the same PL as emulsifier (Table 4), despite the fact that the total quantity of omega-3 fatty acids was higher in the emulsions. Liposomes are hollow spherical particles consisting of a continuous phospholipid bilayer only. The emulsion differs from the liposomes by having the inner space of the spherical droplet filled with marine TAG and the emulsifier, i.e. PL in this case, forms a monolayer.

Assuming that the primary site for iron-mediated oxidation is the PL interphase, the size and character of the interphase is likely to affect the OURs. In liposomes, basically all the PL molecules, i.e. including the ones facing the cavity, are exposed to the aqueous phase, which supplies dissolved oxygen. In addition, the average droplet size in the herring oil emulsions was 10.9 ± 0.1 μm, which is 100 orders of magnitude higher than a typical size of liposomes (0.1 μm) [19]. This means that the total surface area was 100-fold larger for liposomes. This gives the iron atoms a

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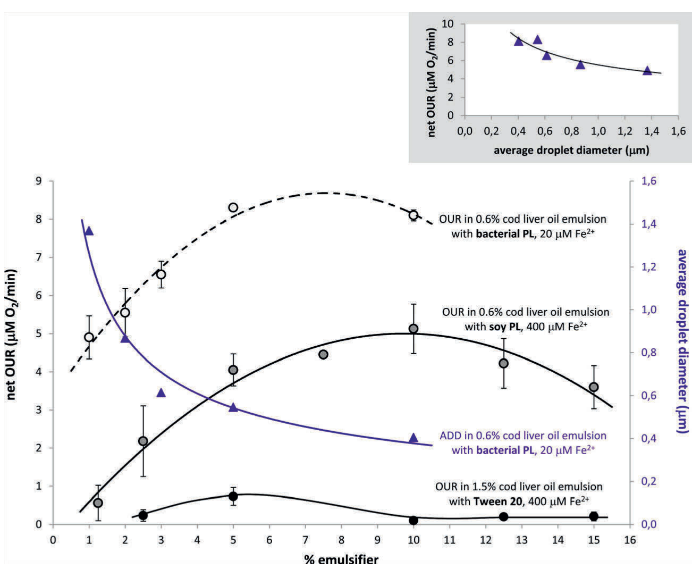

Figure 7. Influence of emulsifier concentration on Fe^{2+}-mediated oxidation in different emulsions, and on the ADD in 0.6% cod liver oil emulsion stabilized with bacterial PL. Inset: Relationship between OUR and ADD in 0.6% cod liver oil emulsion stabilized with bacterial PL.

larger area for encounters with LOOH. In addition, the peroxidation is propagated only within the PL bilayer, meaning the radicals and dissolved oxygen molecules do not need to diffuse deeper into the droplet core, as in case of emulsions, in order to propagate oxidation. These factors could explain higher OUR in the liposomes than in the emulsions.

3.7 Unsaturation of the emulsifier

Experiments where herring roe PL were replaced with soy PL in the 10% herring oil emulsions were performed. The specific OUR rate and the relationship between the different concentrations of Fe^{2+} and the initial drop are given in Table 4.

Iron-mediated oxidation was greatly suppressed in emulsions stabilized by soy PL – the magnitude of the initial drop only slightly increased with increasing concentration of iron and the following linear oxygen consumption was completely inhibited for all the tested Fe^{2+} concentrations (Fig. 5C and D), clearly demonstrated by the negative specific OUR (Table 4).

As discussed in Section 3.5, the pH of the continuous phase has a great impact on iron-mediated oxidation. The pH of the emulsions with herring PL ranged from 3.5 to 4.0 after addition of iron, while the pH of the emulsions with soy PL was initially between 6.1–6.2 and decreased to 5.1–5.7 after addition of iron. In this view, the higher pH in emulsions with soy lecithin would suggest higher susceptibility to iron-mediated oxidation due to better attraction between iron and the surface of the droplets. However, the opposite situation was observed. Therefore, pH can be eliminated as the main influencing factor for this case.

Soy PL contained six times less PUFA than herring roe PL. Unsaturation of the fatty acids has an influence on the structure and permeability of the phospholipid bilayer [8]. Phospholipid membranes containing less unsaturated fatty acids are more rigid and more tightly packed than membranes containing LC PUFA [44], hence possibly more stable against –OOH penetrations into the region of PL heads to come into contact with the iron ions.

Iron was also inactive in 0.9% liposomes from bacterial PL (Fig. 3C). Low degree of unsaturation in bacterial phospholipids could be entirely responsible for the effect, since the majority of fatty acids were saturated and mono-unsaturated (Table 2). The susceptibility to oxidation is greatly reduced in these fatty acids due to the high energy input which is required to abstract a hydrogen atom from carbon atoms on bisallylic methylene positions [9].

However, 0.6% cod liver oil emulsion stabilized with bacterial PL was oxidized by Fe^{2+} (discussed in Sections 3.4–3.6). The net OUR was found to depend on the amount of emulsifier, as shown in Fig. 7, which affected droplet sizes, and that had an impact on iron activity. Therefore, in addition to the unsaturation level of the emulsifier, the ratio between PL (emulsifier), TAG (lipids in the core of the droplets), and the continuous phase (water) in emulsions seems to be important for iron-mediated oxidation.

The outcomes obtained by measurement of dissolved oxygen concentration are in agreement with other studies concluding that the nature and properties of the emulsifier influence lipid oxidation in multiphase systems [8]. In

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emulsions stabilized by PL, the degree of unsaturation of the fatty acids is one of the key parameters for formation of LOOH and thus iron-mediated oxidation.

3.8 Effect of xanthan gum

Oxidation experiments with herring oil emulsions stabilized with herring PL, and with and without xanthan gum, a commercial food thickener, dissolved in the aqueous phase (0.2%) were performed with Fe^{2+} (15 and 30 μM) as a prooxidant.

The initial drop in oxygen concentration as well as the net OUR for Fe^{2+}-mediated oxidation was significantly reduced in the emulsions containing xanthan gum (Fig. 4D and Fig. 8). This could be attributed to the ability of xanthan gum to bind Fe^{2+}, with a reported binding capacity 0.6 mol Fe^{2+}/kg xanthan [49]. The reduction in the initial drop indicates that the binding preference is for Fe^{2+} ions, which is in accordance with a study of Shimada et al. [49]. Xanthan gum therefore acted as an antioxidant/chelator in iron-mediated oxidation and this ability may be advantageous in food.

Based on the value for binding capacity, all the added iron should be chelated, as the xanthan gum was in 40- and 20-fold excess (on a molar basis) of added iron – 15 and 30 μM, respectively. This was however not observed. The activity of iron was reduced by 30 and 59% for the two concentrations, respectively (Fig. 8). The binding capacity may be lowered due to some physicochemical properties of the system – for example pH and ionic strength may interfere [50]. In Section 3.4, it was postulated that iron is bound by phosphate groups within PL heads. Assuming that there is a competition between iron being held by the PL-phosphates and being complexed by the xanthan gum the iron might not be entirely chelated by the xanthan gum.

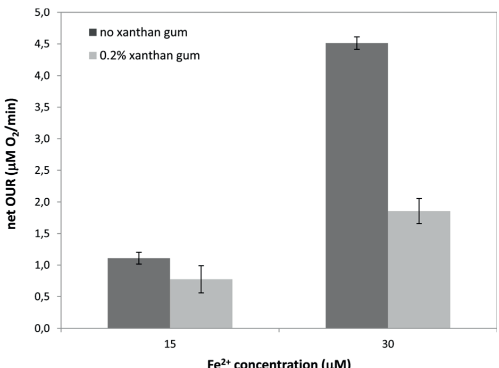

Figure 8. Effect of xanthan gum dissolved in the aqueous phase (0.2%) on OUR in Fe^{2+}-mediated oxidation in 10% herring oil emulsions stabilized with herring PL.

3.9 Effect of chloride content

Chloride anions originating from inorganic salts are common elements in food. In liposomes made from PL from the same species (i.e. either cod or herring), the susceptibility to Fe^{2+}-mediated oxidation was significantly higher for the liposomes lacking chloride anions (Table 4).

Liposomes prepared from PL isolated from the roes prewashed with sea water showed substantially higher zeta potentials than liposomes prepared from PL isolated from the raw roes. The least negative zeta potential value was found for liposomes containing the highest concentration of chloride anions. Addition of 0.1 M NaCl into the aqueous phase (tested at pH 5.5) resulted in an increase in zeta potential for all the liposomes (Table 3). Clearly, the susceptibility to Fe^{2+}-mediated oxidation and the zeta potential values are affected by the presence of chlorides and their concentration.

These data are in accordance with the trend observed in 0.5% cod liver oil emulsions stabilized with bacterial PL. Addition of NaCl (0.0–0.1 M) into the aqueous phase resulted in decreased OUR and increased zeta potential values, as shown in Fig. 9.

In the study of Mozuraityte et al. [30] the following ions added into the aqueous phase of cod roe liposomes did not influence the rate of iron mediated oxidation: Na^+, K^+, Ca^{2+}, Mg^{2+}, SO_4^{2-}, and NO_3^-. On the other hand, chlorides (Cl^-) and dihydrogen phosphate ($H_2PO_4^-$) had a reducing effect on the OUR. The zeta potential was affected conversely: Cl^- did not affect the zeta potential, while the cations had an effect on the surface charge.

It is therefore reasonable to assume that the liposomes in this study did not contain only chlorides, but also the counter cations, analysis of which was not performed, otherwise the zeta potentials would not differ with varying endogenous content of chloride anions.

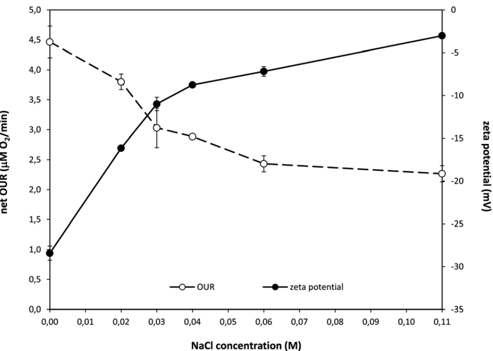

Figure 9. Relationship between the net OURs (empty symbols, μM O_2/min) and zeta potential (full symbols, mV) and different NaCl concentrations (0.0–0.1 M) in 0.5% cod liver oil emulsion stabilized by bacterial PL in oxidation mediated by 7.5 μM Fe^{2+}. The results are mean values of 2–4 parallels ±SD.

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The increase in the zeta potential could be attributed to a tighter compaction of the Na^+ and K^+ ions near the surface of the droplets, resulting in a greater shielding of the negative surface [19]. In this respect, monovalent cations modify the charge due to a shielding effect, making the liposome particles more positive, i.e. more repulsive with respect to iron cations.

While Na^+ ions modified the surface charge of the particles but not the OUR, the Cl^- affected the OUR but not the surface charge. The latter phenomenon remains to be satisfactorily explained. The data suggest that chloride anions interact with iron in the aqueous phase. Nevertheless, both Cl^- and the counter ions play an important role in the decreased susceptibility of liposomes to iron-mediated oxidation in the presence of chloride salts.

In conclusion, the red-ox cycling of iron (Fe^{2+}/Fe^{3+}) upon reaction with pre-formed lipid hydroperoxides (LOOH) located primarily in the emulsifier interface and secondarily in the core of emulsion droplets was the major mechanism in the emulsions and liposomes containing oxidatively sensitive marine lipids. Strong dependency of the pro-oxidant activity of iron on pre-formed LOOH was demonstrated. It is also argued that iron atoms are located within the phospholipid heads, at the level of phosphorous atom, being electrostatically held by negative oxygen atoms on the phosphate groups.

The pH of the continuous aqueous phase affects the surface charge of the lipid droplets stabilized by phospholipids. The highest oxidation rates appeared in various liposomes/emulsions at the pH range 4.5–5.5, making this pH range optimal for iron activity. The low acidity or neutral character of the environment however does not prevent Fe^{2+} from mediating lipid oxidation entirely, presumably due to the association of iron atoms with PL-phosphates.

The pro-oxidant effect of iron was reduced by using less unsaturated emulsifiers (bacterial and soy PL, and Tween 20). The results support a theory that these molecules form a less oxidizable interfacial layer around the oil droplets, due to the low unsaturation level in the fatty acids. In case of Tween, the interface had a character of a barrier between iron and lipid hydroperoxides in the core of the emulsion droplet. The protection by unsaturation of the emulsifier was shown to be dependent on the optimal amount of the emulsifier. The pro-oxidant effect of iron was also reduced by the presence of endogenous chloride anions, and by addition of NaCl or xanthan gum into the systems.

The outcomes of this study obtained by quantification of OURs from dissolved oxygen concentration curves agree or are complimentary with a number of studies where measurements of primary and secondary oxidation products were employed which makes the oxygen uptake method a valid and complementary tool for oxidation studies. The method may be useful for studies on reduction of oxidation of marine lipids in emulsion-based products and screening or optimization of psychochemical and chemical conditions.

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PAPER II

Antioxidant activity of phenolic acids in lipid oxidation catalyzed by different prooxidants

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Antioxidant Activity of Phenolic Acids in Lipid Oxidation Catalyzed by Different Prooxidants

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The antioxidant activity of three naturally occurring phenolic acids, caffeic (CaA), ferulic (FeA), and *p*-coumaric acid (CoA), and a synthetic compound, propyl gallate (PG), was evaluated in a food-related model system, a liposome dispersion of marine polyunsaturated fatty acids. Oxidation was induced by two different prooxidants, free iron ions and bovine hemoglobin (Hb). Continuous measurement of oxygen uptake was used to quantify the rate of lipid oxidation at steady state. Free iron-induced oxidation was reduced in the following order: PG > FeA > CoA. Caffeic acid worked as a prooxidant and increased the oxidation rate by a factor of 9. For Hb-induced oxidation, the relative efficiency was PG > CaA ~ FeA ≫ CoA. The antioxidant activity was also evaluated by four antioxidant capacity assays. In the Folin−Ciocalteu, ferric reducing/antioxidant power, and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays, the antioxidant activity followed the sequence PG > CaA > FeA > CoA. The order for the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay was found to be PG > CoA ~ FeA > CaA. The assays mainly reflected reducing abilities of the compounds. This work reports that in addition to the differences in the chemical structure of antioxidants, the antioxidant activity of phenolic compounds depends also upon the type of prooxidant.

KEYWORDS: Phenolic antioxidants; lipid oxidation; iron; hemoglobin; oxygen uptake

INTRODUCTION

Foods containing long-chain *n*-3 polyunsaturated fatty acids (LC-PUFA) are especially labile with respect to oxidation, which causes formation of undesirable flavors and rancid odors, production of potentially toxic compounds, and loss of the health beneficial and essential fatty acids. Prooxidative agents, such as transition metals (iron, copper) or heme pigments (hemoglobin, myoglobin), significantly promote quality loss and reduce the shelf life of PUFA-rich foods. Iron ions and hemoglobin can be found as endogenous constituents in both raw fish materials and a wide variety of seafood products.

One industrially acceptable technique to control oxidative instability of fatty products is the application of antioxidative compounds with different mechanisms of action, including radical scavengers, singlet oxygen quenchers, photosensitizer inactivators, and metal chelators. Conventionally used antioxidants include synthetics, such as propyl gallate (PG), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ), or ethylenediaminetetraacetic acid (EDTA), and nature-identical compounds, such as L-ascorbic, citric, and tartaric acids; natural antioxidants found in rosemary and tocopherol extracts are also commercially available (*1*).

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There has been a growing trend among consumers to prefer foods without synthetic additives, which has resulted in considerable worldwide attention to replacement of fully synthetic food antioxidants with naturally occurring ones. Unfortunately, the number of antioxidative compounds of natural origin approved by authorities is very small. It is therefore challenging as well as necessary to investigate the potential usefulness of other natural compounds, both individually and in mixtures. (*2*) During the past two decades, there has been intensive research on plant phenolics as suitable protectants against oxidation. Hydroxycinnamic acid derivatives have gained special attention owing to their abundant occurrence in a wide variety of fruits, vegetables, cereals, and cocoa and coffee beans (*3*, *4*).

The evaluation of the antioxidant activity of the different phenolic compounds has lately become an important research issue, and two general approaches have been applied: (i) an indirect approach by means of so-called *antioxidant capacity* (AOC) *assays* and (ii) a direct approach by use of lipid model systems (*5*).

The direct approach utilizes a multiplicity of lipid model systems: bulk oils, biphasic systems, emulsions, membrane structures [liposomes, microsomes, low-density-lipoproteins (LDL), intact cells], as well as a variety of PUFA-rich foods. A large number of these studies have shown that phenolics can act both antioxidatively and prooxidatively depending upon the

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physicochemical nature and the composition of the lipid system as well as antioxidant structure and concentration (*3*, *6−12*).

To select the right antioxidant for a given application, understanding the basic factors that affect the activity of phenolics in lipid oxidation is therefore beneficial. The choice of a food-related model system for investigative purposes is somewhat problematic. Real foods and isolated membranes (microsomes, LDL) mostly contain a variety of endogenous components, which may be difficult to control and which may interfere with the effects of added antioxidants. On the other hand, the model system should not be oversimplified. It should be transparent with respect to its compositional characterization and devoid of any interfering reactions that can complicate interpretation of the experimental results and drawing conclusions (*13*).

Lipid oxidation is conventionally studied by determination of peroxide value (PV), thiobarbituric acid reactive substances (TBARS), conjugated dienes, or anisidine value (AV) or by assessing volatile compounds. Peroxides are primary products in the oxidative breakdown of lipids, and their formation is the net result of the production rate and the decomposition rate (*1*). As the decomposition rate of peroxides is a function of at least pH and temperature, the production rate is difficult to quantify. The end products of lipid oxidation are the result of several reaction pathways from peroxides; these reactions do not follow one line, but several breakdown lines are possible, giving different end products from the same peroxide molecule. Analyzing only one or a few final oxidation compounds might therefore be misleading. An alternative approach is to focus on measurements of changes in oxygen, one of the lipid oxidation substrates, which has been employed in several studies, including earlier studies in our laboratory (*14−16*).

An increased interest in antioxidant activity has led to the development of a wide array of indirect methods to measure the antioxidant capacity (*5*, *17*). Different results have been obtained both between individual methods and within a method itself. Moreover, many inherent drawbacks in these assays have been found and discussed in a number of works (*5*, *17−20*). Despite these criticisms, AOC assays are still being routinely used for the evaluation of antioxidant capacity by food laboratories (*21*), although there have been efforts to improve procedure protocols of the existing methods (*22*), as well as efforts to develop new assays utilizing and combining different approaches (*23−26*).

The objective of this study was to evaluate the antioxidant activity of three naturally occurring phenolic acids that have potential as food antioxidants, caffeic acid (CaA), ferulic acid (FeA), and *p*-coumaric acid (CoA), and one synthetic phenolic antioxidant, propyl gallate (PG). A food-related lipid model system, liposome dispersion of long-chain PUFAs in marine phospholipids, was chosen to avoid the complexity of real food matrices. Lipid oxidation was induced by two different dietary prooxidants, free iron (Fe^{2+}, Fe^{3+}) and bovine hemoglobin (Hb), as these prooxidants differ substantially in prooxidative mechanism (*1*, *27*). Continuous measurement of oxygen uptake was used to quantify the rate of lipid oxidation. In addition, four commonly used spectrophotometric antioxidant capacity assays were used for comparison and additional information on the antioxidant capacity of the studied compounds.

MATERIALS AND METHODS

Materials. Cod roe from Pacific cod (*Gadus macrocephalus*) caught in the North Pacific Ocean was used for extraction of phospholipids. The cod roe was frozen at −40 °C until needed.

Propyl gallate, caffeic acid, ferulic acid, *p*-coumaric acid, 2.0 M Folin−Ciocalteu phenol reagent, 2-(*N*-morpholino)ethanesulfonic acid (MES), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), 2-methylpentane, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), sodium carbonate, sodium acetate, bovine hemoglobin, sodium sulfite, trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), potassium persulfate, and catalase from bovine liver were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid, acetic acid, potassium chloride, formic acid, ferric chloride tetrahydrate, ammonium thiocyanate, titrisol [$(NH_4)_2Fe(SO_4)_2$], ferrous sulfate heptahydrate, and all of the solvents used were supplied by Merck KGaA (Darmstadt, Germany). Anhydrous ferric chloride and IDRANAL II (ethylenediaminetetraacetic acid) was purchased at Riedel de Haën (Seelze, Germany). Sodium hydroxide and ferrous chloride tetrahydrate were obtained from Fluka Chemie (Buchs, Switzerland). Nitrogen (N_2) gas (99.999%) was provided by AGA AS, Oslo, Norway. All chemicals and solvents were of analytical grade.

Isolation of Phospholipids. The cod roe was allowed to thaw overnight at 4 °C. The extraction of total lipids from cod roe was performed according to the method of Bligh and Dyer (*28*). The phospholipids (PL) were isolated from the total lipids using the acetone precipitation method, as described by Kates (*29*) and modified by Mozuraityte et al. (*14*). The phospholipids dissolved in chloroform were stored in the dark at −20 °C until needed.

Purity of Phospholipids. The composition of isolated phospholipids was determined by thin layer chromatography (*30*) and detected by a Iatroscan thin layer chromatography−flame ionization detector system (TLC-FID analyzer TH-10 MK-IV, Iatron Laboratories, Inc., Tokyo, Japan) as described by Mozuraityte et al. (*14*). Three analyses were performed, and the results were expressed in area percentage as the mean value ± standard deviation (SD).

Phospholipid Classes. The classes of isolated PL were analyzed by P-31 NMR. Fifty milligrams of dried mass of phospholipids was dissolved in a 0.6 mL solution of chloroform-*d*/methanol-*d* (2:1, v/v) containing the internal standard (triethylphosphate) in 5 mm NMR tubes. NMR spectra were recorded on a Bruker Avance DPX 300 spectrometer with QNP probe operating at a P-31 frequency of 121.49 MHz at ambient temperature (25 °C). The acquisition parameters used were as follows: spectral width, 30 ppm; 20K time domain data points; zero-filled to 64L; acquisition time, 2.8 s; relaxation delay, 50 s; 90° acquisition pulse. Chemical shifts were referred to triethylphosphate ($\delta = 0$ ppm).

Fatty Acid Composition. The fatty acid composition of the phospholipids was determined by gas chromatography (GC) of their fatty acid methyl esters as described by Dauksas et al. (*31*) The lipids were transesterified and extracted into hexane according to AOCS method Ce 2-66 (*32*). Free fatty acid methyl esters were identified by comparison of their retention times with those of the reference solution (GLS-68D; Nu-Chek-Prep) chromatographed under identical GC conditions. Two replicate analyses were performed and the results were expressed in GC area percent as a mean value ± SD (standard deviation).

Peroxide Value (PV). PV was analyzed by the ferric thiocyanate method as described by the International Dairy Federation (*33*) and modified by Ueda et al. (*34*) and Undeland et al. (*35*). The analysis was performed in triplicate.

Analysis of Thiobarbituric Acid Reactive Substances (TBARS). TBARS values were determined according to the spectrophotometric method described by Ke et al. (*36*). The absorbance values of samples were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations (μM/g of fat). The analysis was performed with five parallels.

Preparation of Liposomes. The liposome dispersion was prepared according to the method of Mozuraityte et al. (*14*) fresh before experiments. A chloroform solution of phospholipids was evaporated to dryness with a stream of nitrogen gas (99.99%), and the residual solvent was completely evaporated under vacuum (2 h). The dried mass of phospholipids was dissolved in a 5 mM MES buffer, pH 5.5 or 3.0, to a concentration of 30 mg/mL, and the solution was sonicated with Vibra Cell (Sonics & Materials Inc.). MES buffer was used because it does not bind iron into complexes, has a very low solubility in nonpolar solvents, and has $pK_a = 6.1$, which is suitable for our oxidation experiments, as most of them were performed at pH ∼5.5. At higher concentrations of MES buffer, a higher rate of Fe^{2+} autoxidation was observed. During and after the sonication the phospholipids were kept on ice. For all of the

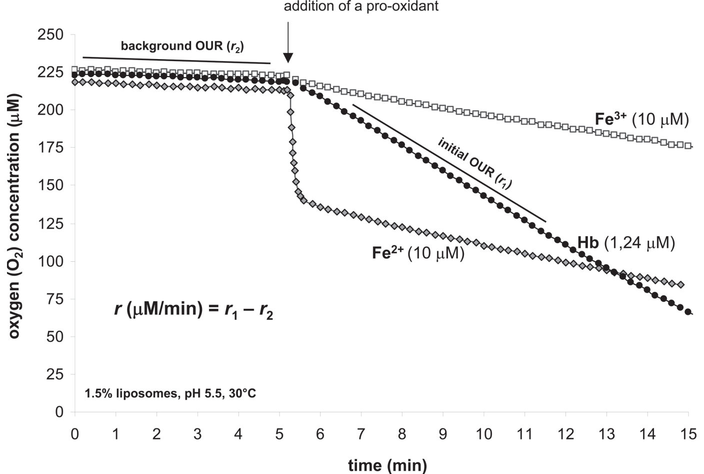

Figure 1. Representative oxygen uptake curves of oxidation of polyunsaturated fatty acids in liposomes (1.5%, pH 5.5, 30 °C) recorded before and after addition of different prooxidants (noninhibited oxidation): (◆) 10 μM Fe^{2+}; (□) 10 μM Fe^{3+}; (●) 1.24 μM Hb. Calculation of oxygen uptake rate (OUR) is schematically depicted in the curve for Hb.

experiments, the liposome solution was further diluted with a MES buffer to a concentration of 15 mg/mL.

Oxidation Experiments. The rate of oxidation was quantified from the rate of disappearance of the dissolved oxygen in the reaction mixture (1 mL). A polarographic oxygen electrode (Oxygraph system, Hansatech Instruments Ltd., Norfolk, U.K.) was used to continuously measure the dissolved oxygen concentration. Each oxygraphic cell was closed with a plunger with a capillary opening, equipped with a magnetic stirrer, and thermostated at 30 °C.

Stock solutions of PG, CaA, FeA, and CoA were prepared in 96% ethanol and stored in the dark at 4 °C for a maximum of 14 days. Working solutions were prepared daily by diluting an appropriate aliquot of the stock solution with ethanol. Stock solutions of Fe^{2+} ($FeSO_4 \cdot 7H_2O$) and Fe^{3+} ($FeCl_3$) in 0.5 M HCl were prepared monthly. Working solutions of Fe^{2+} and Fe^{3+} were prepared daily by diluting an appropriate aliquot of the stock solution with MES buffer; the pH was kept at 2.0 to maintain iron solubility. Working solution of bovine hemoglobin (Hb) was prepared fresh before experiments by dissolving Hb in 5 mM MES buffer (pH 5.5). The concentrations of prooxidants in the reaction mixture were 10 μM Fe^{2+}, 10 μM Fe^{3+}, and 20 μg/mL Hb, the latter corresponding to 1.24 μM iron donated via Hb.

The curves of dissolved oxygen concentration as a function of time were recorded. A background oxygen uptake rate (OUR) was measured for 2−5 min before the addition of a sample (antioxidant or ethanol). After sample addition, a background OUR (r_2) was observed again, usually for 5−10 min. When a steady background OUR was reached, a prooxidant (Fe^{2+}, Fe^{3+}, or Hb) was added into the dispersion to initiate oxidation and the initial OUR of oxidation (r_1) was measured, that is, the oxygen consumption rate between the second and fourth minutes after addition of a prooxidant. The rate of oxidation (r) was found by subtracting the initial OUR from the background OUR ($r = r_1 - r_2$), as illustrated on the Hb curve in **Figure 1**. Inhibition (%) in relation to the respective noninhibited oxidation (added ethanol) was calculated to evaluate the antioxidant effects: $I(\%) = 100 - [(r_{inh}/r_{non}) \times 100]$. Two or three parallel cells were run for each experiment. The pH of the liposome solutions was verified after each experiment by a Philips pH meter (model PW 9420, Pye Unicam, Cambridge, U.K.) coupled with a glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, NV).

The presence of ethanol (maximum 2% in a reaction volume) that was used to dissolve the phenolics did not have any significant influence on the oxygen uptake by liposomes themselves (data not shown) or on the prooxidative activity of Fe^{2+} and Fe^{3+} ions (6.0 ± 0.4 and 7.0 ± 0.6 μM O_2/min, respectively) and Hb (presence of ethanol, 20.8 ± 1.1; absence of ethanol, 19.4 ± 0.8 μM O_2/min).

Oxygraph software "oxyg32" and MS Excel was used for data processing and statistical analysis. The significance level was set at 95% ($p = 0.05$).

For the antioxidant capacity assays, a 10 mM methanolic stock solution of each compound was prepared and stored in the dark at 4 °C for a maximum of 14 days. Working solutions were prepared daily by dilution of suitable aliquots of a stock solution with methanol.

Folin−Ciocalteu (FC) Assay. The FC assay was performed as described by Singleton et al. (*37*) with some modifications (*38, 39*). Briefly, a series of 0−3 mM working solutions of PG and CaA and 0−5 mM FeA and CoA were prepared. Deionized water (10 mL), antioxidant solution (1 mL), and 2.0 M Folin−Ciocalteu phenol reagent (1 mL) were transferred to a 20 mL volumetric flask, the reaction mixture was mixed by shaking, and after 3 min, 2 mL of 25% Na_2CO_3 solution (75 g/L) was added. The volume was brought up with deionized water. The absorption at 725 nm was read after 1 h of incubation at room temperature. Water was used as a blank. Five point graphs of antioxidant concentration versus absorbance values were constructed, and the FC value was taken as the slope of the linear curve derived from the constructed graphs. The assay was carried out three times with each compound, and the average slope value ± standard deviation (SD) was calculated.

FRAP Assay. The FRAP assay was performed as described by Benzie et al. (*40*) and adapted by Nenadis et al. (*38*). For the analysis, a series of 0−150 μM working solutions of PG and CaA, 0−200 μM FeA, and 0−2700 μM CoA were prepared (final dilution in the reaction mixture 1:15). Five point graphs of antioxidant concentration in the reaction mixture versus ΔA ($\Delta A = A_{AH} - A_{cont}$) were constructed. The FRAP value was taken as the slope of the linear curve derived from the constructed graphs. The assay was performed three times with each compound, and the average slope value ± SD was calculated.

DPPH Assay. The DPPH assay was performed as described by Brand-Williams et al. (*41*) with some modifications (*19, 38, 42*). Briefly, the day before analysis, 0.1 mM methanolic DPPH$^{\bullet}$ working solution was prepared and kept on a magnetic stirrer overnight at 4 °C. A series of 0−750 μM methanolic working solutions of PG, 0−1200 μM CaA, and 0−2400 μM FeA were prepared fresh from stock solutions (final dilution in the reaction mixture 1:30). An aliquot of DPPH$^{\bullet}$ solution (2.9 mL) was mixed with 0.1 mL of a sample or methanol (blank) and vortexed well. After 20 min of incubation at room temperature, the absorbance at 515 nm was recorded. Water was used as a blank. Five point graphs of inhibition (%) of initial absorbance of the DPPH$^{\bullet}$ solution [$I(\%) = (1 - A_{sample}/A_{blank}) \times 100$] versus antioxidant concentration in the reaction mixture were constructed, and EC_{50} values were calculated from the linear curves derived from the constructed graphs. The assay was performed two times with each compound, and the average EC_{50} value ± SD was calculated.

Table 1. Characterization of Phospholipids Isolated from Cod Roe

phospholipid classes (%)	phosphatidylcholine (PC)	69
	phosphatidylethanolamine (PE)	23
	lyso-PC	5
	lyso-PE	3
lipid classes (%)	phospholipids	97.9 ± 1.2
	free fatty acids	0.4 ± 0.1
	cholesterol	1.0 ± 0.5
	monoacylglycerol	1.0 ± 0.7
	unknown	<1.0
fatty acids (%)	saturated	28.2 ± 0.8
	monounsaturated	25.8 ± 0.3
	polyunsaturated	46.0 ± 0.5
	20:5 n-3 (EPA)	14.2 ± 0.2
	20:6 n-3 (DHA)	29.8 ± 0.3
	other PUFA	2.0 ± 0.0
PV (mequiv of peroxide/kg of fat)		6.6 ± 1.3
TBARS (μM/g of fat)		2.4 ± 0.2

ABTS Assay. The ABTS assay was performed as described by Nenadis et al. (*43*) with a few modifications: ethanol was replaced with methanol, and the amount of sample added to the $ABTS^{\bullet+}$ solution was 200 μL. For the analysis, a series of 0−55 μM working solutions of PG and 0−110 μM CaA, FeA, and CoA were prepared fresh from stock solutions (final dilution in the reaction mixture 1:11). Five point graphs of inhibition (%) of initial absorbance of the $ABTS^{\bullet+}$ solution [$I(\%) = (1 - A_{sample}/A_{blank}) \times 100$] vs antioxidant concentration in the reaction mixture were constructed, and EC_{50} values were calculated from the linear curves derived from the constructed graphs. The assay was performed twice with each compound, and the average EC_{50} value ± SD was calculated.

To compare the antioxidant activities, the absolute values for each antioxidant and each assay were recalculated into propyl gallate equivalents, the absolute value for PG being equal to 1.

RESULTS AND DISCUSSION

Characterization of Cod Roe Phospholipids. The phospholipid classes, lipid classes, and fatty acid profile of the phospholipids (PL) isolated from cod roe, which were used for preparation of liposomes, are presented in **Table 1**. To evaluate the oxidation level of the material, PV and TBARS were measured after isolation (**Table 1**). Both PV and TBARS values indicated a low degree of oxidation; the PV value was consistent with the PV values for our previous isolations (*14*, *44*).

Inhibition of Iron-Catalyzed Oxidation. Both Fe^{2+} and Fe^{3+} were used as promoters of lipid oxidation. The time curves of oxygen uptake after addition of each prooxidant into liposomes (referred to as *noninhibited* oxidation) are shown in **Figure 1**. In our previous paper it has been explained that the initial fast consumption of dissolved oxygen after the addition of Fe^{2+} is due to oxidation of Fe^{2+} to Fe^{3+} by pre-existing peroxides. This process generates alkoxy and peroxy lipid radicals, which rapidly deplete dissolved oxygen by forming lipid hydroperoxides. Once the equilibrium between Fe^{2+} and Fe^{3+} is achieved, redox cycling of iron takes place, resulting in a constant rate of oxygen consumption observed after the initial drop, where Fe^{3+} reduction is the rate-limiting factor (*15*).

The relative efficiency of the tested phenolics (**Figure 2**), compared at 200 μM concentration, had the same trend regardless of the state of iron: PG was the most powerful compound, followed by FeA; CoA did not show any significant inhibitory effects. The inhibition (%) of oxidation as a function of PG, FeA, and CoA concentration is shown in **Figure 3**. Conversely, CaA strongly enhanced oxidation. The increase (%) in oxidation for different concentrations of CaA is shown in **Figure 4**. The similar values for Fe^{2+} and Fe^{3} for both inhibited and promoted oxidation clearly show that behavior of the tested phenolics is not dependent upon the initial state of iron, which supports the theory of redox cycling of iron (*15*).

In the tested concentration range of PG (1−200 μM), only the concentrations above 10 μM (PG/Fe ≥ 1) were efficient in inhibiting the oxidation rate. The concentration of 200 μM

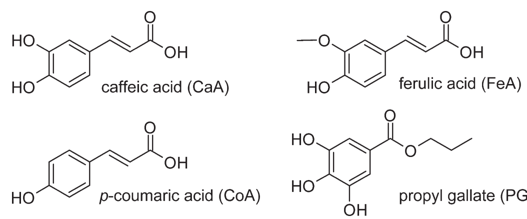

Figure 2. Molecular structures of three naturally occurring phenolic antioxidants, caffeic acid, ferulic acid, and *p*-coumaric acid, and a synthetic phenolic compound, propyl gallate.

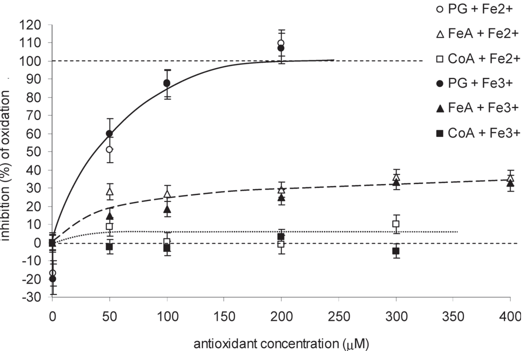

Figure 3. Inhibition (%) of Fe^{2+} (10 μM) and Fe^{3+} (10 μM) catalyzed oxidation of polyunsaturated fatty acids in liposomes (1.5%, pH 5.5, 30 °C) by different concentrations of propyl gallate (PG), ferulic acid (FeA), and *p*-coumaric acid (CoA). The values are given as the means of two to five parallel experiments ± standard error (SE). Positive values represent inhibition of oxidation, whereas negative values represent an increase in oxidation.

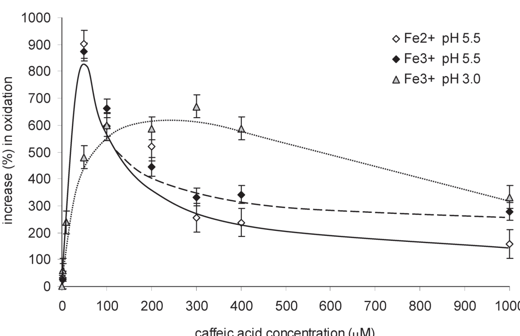

Figure 4. Increase (%) in Fe^{2+} (10 μM) and Fe^{3+} (10 μM) catalyzed oxidation of fatty acids in liposomes (1.5%, 30 °C) at pH 5.5 and 3.0 by different concentrations of caffeic acid (CaA). The values are given as the means of two to five parallel experiments ± standard error (SE).

Figure 5. Time curves of oxygen consumption in Fe^{3+}-induced oxidation (line a), and Fe^{3+}-induced oxidation inhibited by propyl gallate (line b) or promoted by caffeic acid (line c).

completely inhibited oxidation. When 1 μM PG concentration was tested (PG/Fe = 0.1), an increase in oxidation rate both in Fe^{2+} and in Fe^{3+} catalyzed oxidation (20 ± 8 and 17 ± 7%, respectively) was observed.

CaA strongly enhanced oxidation at all tested concentrations except for the lower concentration (0.1 μM), at which CaA did not have any significant effect on the oxidation rate. The prooxidant effect of CaA showed a maximum at 50 μM (CaA/Fe ≥ 5) at pH 5.5 and at 200 μM (CaA/Fe ≥ 20) at pH 3.0. At higher CaA concentrations the prooxidant effect decreased. The time course of oxygen consumption for selected concentrations of PG and CaA after addition of Fe^{3+} is shown in **Figure 5**.

FeA (50−400 μM) was capable of inhibiting free iron promoted oxidation at all levels of addition. The degree of inhibition did not markedly increase with increasing concentration as was observed with PG. FeA was found to be a better antioxidant than *p*-coumaric acid; however, among the tested compounds FeA could be characterized as a less potent antioxidant.

All of our oxidation experiments were performed in the same way to observe the inhibition of the very onset of iron-induced oxidation, with the antioxidant already present in the liposome solution (**Figure 5**). However, when the order of addition of an antioxidant and a prooxidant was reversed, that is, antioxidant was added into already initiated oxidation, a significant decrease (or increase for CaA) in oxygen consumption was observed as well (data not shown). In our earlier study it was proposed that one redox cycle of iron generates a constant number of lipid alkoxy and peroxy radicals as a result of decomposing lipid hydroperoxides, which leads to a constant rate of oxygen consumption (*15*); thus, the system reaches a steady state. The experiments show that it is possible to reduce the rate of iron-catalyzed oxidation from the very beginning as well as at the later stages by eliminating the generated radicals by means of the addition of antioxidants.

Caffeic Acid and Iron-Catalyzed Oxidation. The prooxidative behavior of CaA in the free iron catalyzed oxidation is most likely the result of its ability to reduce Fe^{3+}, which was verified by the FRAP assay (shown below).

An intramolecular electron transfer (IET) within a temporary CaA−Fe^{3+} complex has been reported to be responsible for the reduction of ferrous ions (Fe^{3+}) by CaA (*45*). The reaction releases Fe^{2+} and produces *o*-semiquinone radical and possibly *o*-quinone (**Figure 6**). Superoxide anion ($O_2^{\bullet-}$) has been proposed to be generated from triplet oxygen during the IET reaction (*46*).

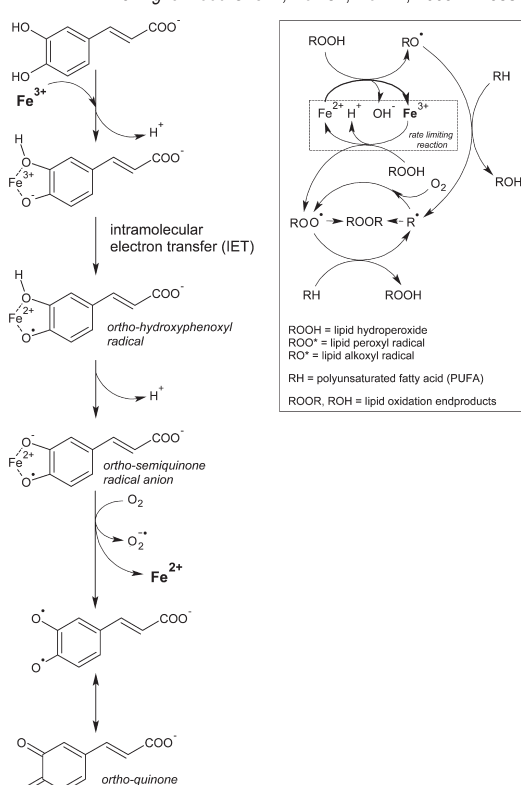

Figure 6. Reduction of ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron by intramolecular electron transfer within a caffeic acid molecule (modified from ref *46*). (Inset) Low molecular weight (free) iron catalyzed lipid oxidation (modified from ref *14*).

There are several possible pathways for its further reactions. In an aqueous medium, $O_2^{\bullet-}$ is capable of Fe^{3+} reduction, forming hydrogen peroxide (H_2O_2) or triplet oxygen ($^3O_2$) (*1*). Measurable amounts of hydrogen peroxide were not found when catalase (40 μM), an enzyme decomposing hydrogen peroxide into water and oxygen molecules, was added into liposomes containing iron and CaA, as dissolved oxygen concentration did not increase significantly after the addition of catalase (data not shown).

In experiments with a ratio of CaA/Fe ≤ 1, the reaction pathways of CaA−Fe complex formation and decomposition may differ. When iron is present in great abundance, a total breakdown of CaA rather than formation of quinones was reported to follow complex formation (*45*). This could explain the markedly lower prooxidative activity at 1 μM CaA concentration and no effect at 0.1 μM CaA concentration.

Interestingly, the prooxidative activity of CaA was significantly lowered also at 1000 μM CaA concentration, at both pH 5.5 and 3.0. This could be attributed to the radical scavenging abilities of the proportion of CaA that was associated with the phospholipid bilayers or to a low solubility of CaA in aqueous phase.

In an aqueous dispersion of liposome, the stability of hydrated iron ions is sensitive to the pH of the surrounding environment. In aqueous solutions the solubility of iron is maintained by low pH (≤2.0); an increase in pH leads to precipitation of iron as iron

Table 2. Antioxidant Activities of the Studied Phenolic Compounds Tested with Different Antioxidant Capacity Assays Expressed in Propyl Gallate Equivalents[a]

antioxidant	FC assay (slope ± SD)	FRAP (slope ± SD)	DPPH (EC_{50} ± SD)	ABTS (EC_{50} ± SD)
propyl gallate *absolute values*	0.34 ± 0.02	0.119 ± 0.005	10.6 ± 0.3	20.0 ± 0.4
propyl gallate	1.00 a	1.00 a	1.00 a	1.00 a
caffeic acid	0.96 a	0.65 b	0.47 b	0.32 b
ferulic acid	0.30 b	0.54 c	0.22 c	0.43 c
p-coumaric acid	0.11 c	0.04 d	< 0.02 d	0.44 c

[a] Folin−Ciocalteu (FC) assay; ferric reducing/antioxidant power (FRAP) assay; 2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH) assay; 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay. The absolute values are given as means ± SD (standard deviation) of two (DPPH and ABTS) or three (FC and FRAP) parallel measurements; slope = slope value of a linear curve derived from the dependence absorbance = *f*(antioxidant concentration in the reaction mixture); EC_{50} = efficient antioxidant concentration (μM) for scavenging 50% of the radical. Values within the same column with different letters are significantly different at $p < 0.05$.

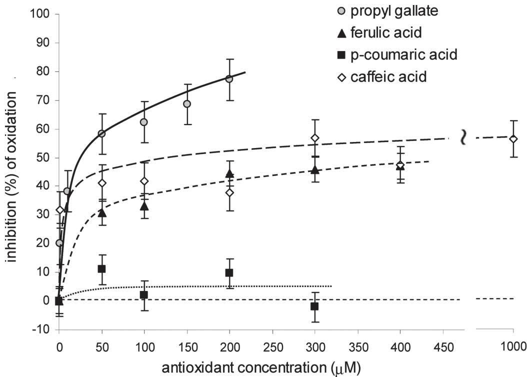

Figure 7. Inhibition (%) of Hb (1.24 μM) catalyzed oxidation of liposomes (1.5%, pH 5.5, 30 °C) by different concentrations of the tested phenolics. The values are given as the means of two to five parallel experiments ± standard error (SE).

hydroxides (*47*). The solubility product constants for Fe^{2+} and Fe^{3+} hydroxides are 5×10^{-17} and 3×10^{-39}, respectively (*15*). Observations in our earlier study (*15*) indicate that in working solutions of iron with a higher pH, formation of iron hydroxides leads to changes in concentration of active iron resulting in somewhat lowered and different OURs between Fe^{2+} and Fe^{3+} mediated oxidation. Thus, both the Fe^{2+} and Fe^{3+} working solutions were prepared at pH 2.0 to prevent hydroxide formation prior to the addition of iron into the liposome solution. We cannot exclude that precipitation of iron occurs in the liposome solution. However, the low concentration of iron (10 μM) and not significantly different OUR values measured in Fe^{2+} and Fe^{3+} initiated oxidation, (7.2 ± 0.4 and 7.0 ± 0.4 μM O_2/min, respectively) suggest that phospholipids attract iron in the way that prevents hydroxide precipitation, as the proportion of active iron remains equal for both Fe^{2+} and Fe^{3+} after redox cycle equilibrium is achieved. The addition of an acidic working solution of iron (10 μL) into liposomes had only a minor effect on the pH value of the resulting reaction mixture, being in the range of 5.3−5.5.

Inhibition of Hb-Induced Oxidation. Except for CoA, all of the tested phenolics inhibited Hb-induced oxidation (**Figure 7**). The relative efficacy, compared at 100 μM concentration, followed the sequence PG > CaA ~ FeA.

The inhibitory effect of PG (1−200 μM) increased strongly with increasing concentration. Contrary to free iron catalyzed oxidation, CaA did not show any prooxidant activity and was able to inhibit oxidation at concentrations above 0.1 μM to 1000 μM; at 0.1 μM CaA was inactive. Slightly increasing inhibitory effect was observed with increasing concentration. FeA was tested in the concentration range from 50 to 400 μM. The degree of inhibition did not markedly increase with increasing concentration, and the inhibitory effect was approximately equal to that of CaA. CoA was inactive toward Hb-induced oxidation at all tested concentrations (50−300 μM) as well as being inactive toward free iron induced oxidation.

Both prooxidants, free iron and Hb, are dispersed in the aqueous phase of the liposome solution. This shows that there has to be attractive interactions between the prooxidants and charged liposome particles, which locate the prooxidants to the very proximity of the phospholipid bilayers. The aqueous environment also allows the prooxidants to be in direct contact with the phenolic molecules distributed in the water phase.

In most of our experiments the molar concentrations of phenolics were > 10 times higher than the concentration of iron in Hb (1.24 μM). However, at molar PG-to-Hb ratio close to 1, PG was efficient in inhibiting oxidation (the inhibition reached 20 ± 4%) (**Figure 7**), which is in contrast with Fe-induced oxidation, for which at the same ratio a prooxidative tendency of PG was observed (**Figure 3**).

So far, the mechanism for Hb-initiated peroxidation has not been fully clarified. Forms of heme-proteins (Hb, Mb) containing the oxoferyll complex (Fe^{4+}=O) are believed to be the main driving force of heme-iron-initiated lipid oxidation (*27*, *48*). The mechanism of phenolics for inhibiting lipid oxidation promoted by Hb has not been elucidated either. Pazos et al. suggested that the mechanism did not seem to be related to a direct effect of phenolics on Hb autoxidation (Hb−Fe^{2+} ⇔ Hb−Fe^{3+}) (*8*).

The studied phenolics are capable of noncovalent binding to proteins (*49*). Binding of chlorogenate to ferrylmyoglobin (Mb−Fe^{4+}) led to reduction of the oxoferryl moiety to a less prooxidative metmyoglobin (Mb−Fe^{3+}) (*50*). Metmyoglobin/H_2O_2-dependent oxidation of LDL also resulted in reduction of ferrylmyoglobin to metmyoglobin by phenolic acids (*51*).

It could be assumed that in the aqueous phase of the liposome solution the phenolic acids can easily enter the heme crevice of Hb and/or bind to ferrylhemoglobin (Hb−Fe^{4+}), where they can quickly reduce the oxoferryl moiety, which is observed as the inhibition of Hb-catalyzed oxidation. Indeed, the studied phenolics with a higher reduction potential inhibited the oxidation more strongly that those with a lower reduction potential.

Due to low polarity and relatively high affinity of PG toward phospholipid membranes (discussed below), PG probably functions both as an efficient free lipid radical scavenger and as a powerful reductant of ferrylhemoglobin, providing the highest inhibitory effects. The inhibitory effect of FeA on Hb-promoted oxidation was approximately equal to that of CaA, although the standard reduction potential of FeA has been reported to be half that of CaA (*52*). Apart from the ability of phenolics to reduce the prooxidative initiator, the accessibility of antioxidant molecules to the heme crevice or the strength of noncovalent bonds between proteins and the phenolics may play an important role.

Antioxidant Capacity Assays. The results from the antioxidant capacity assays are summarized in **Table 2**. In the FC, FRAP, and

DPPH assays, PG showed the highest antioxidant activity followed by CaA, FeA, and CoA. In the ABTS assay, PG was also found to have the highest activity, followed by CoA and FeA, whereas CaA was found to have the lowest antioxidant activity. The obtained orders are in agreement with data reported in the literature (*10–12*, *19*, *43*, *53*, *54*). Although the orders determined by the FC, FRAP, and DPPH assays are almost identical, indirect expression by means of propyl gallate equivalents revealed considerable differences in the degree of activity.

The inconsistencies in the orders and degrees could be attributed to a number of factors, involving some specific reactions between the different assay reagents and the antioxidants, unrelated reactions of phenolics (dimerization, polymerization) that probably occur in the reaction mixtures, and drawbacks and limitations in the chemistry and methodology of the assays (*5*, *17–20*).

Good correlations were found between the orders established in FC, FRAP, and DPPH assays and the order established in Hb-induced oxidation. The order found in the ABTS assay correlated neither with free iron catalyzed oxidation nor with Hb-catalyzed oxidation.

Results from the FC and FRAP assays show that PG and CaA possess a high ability to donate an electron. When related to the catalytic role of metals in lipid oxidation, a positive reducing capacity signals possible redox reactions with transition metals (Fe, Cu) and their reduction into a more prooxidative valence status. The FRAP assays also showed that all of the compounds possess an electron-donating ability in an acidic (pH 3.6) aqueous solution. The oxidative potentials (E_{pa}) for CaA, FeA, and CoA were reported to be 0.212, 0.430, and 0.583 (V vs Ag/AgCl), respectively (*52*), which is in agreement with the orders found in the two assays.

The results support the concepts of other authors that the AOC determined by these assays cannot universally predict the antioxidant activity and should, therefore, serve as a tentative or preliminary estimation of antioxidant capacity; any predictions regarding protection of lipid systems (foods) are uncertain or could even be misleading (*17*). From the reaction mechanism point of view, the assays only provided information on the reducing potentials (ability to donate an electron) of the compounds or the ability to scavenge synthetic free radicals in an aqueous environment via single electron transfer.

Factors Affecting the Antioxidant Activity. The polarity of phenolic molecules has a strong influence on their location in lipid systems (*1*). In emulsion type systems, the affinity of antioxidants toward the interface, represented by phospholipids in our system, is a key parameter, as well.

When PG was added to the liposome dispersion not containing a prooxidant, a total inhibition of the background oxygen uptake by liposomes was observed (data not shown), which clearly indicated that PG was active in the phospholipid bilayers. It should be noted that none of the tested phenolic acids reduced the background oxygen uptake after addition. Another laboratory reported high partitioning of PG in the emulsion interface consisting of egg yolk phospholipids (*55*); microsomal phospholipid membranes incorporated 52.1% of added PG (*8*), whereas 10% of PG was found in liposome bilayers (*56*). On the basis of our observations and these studies, it could be assumed that a substantial amount of PG would be located within the phospholipid bilayers, where it can act as a potent radical scavenger.

On the contrary, partitioning of polar compounds in the oil phase of both biphasic systems and emulsions has been reported to be low in general (*12*, *55*, *57*). According to Medina et al. (*11*), the polarity of the phenolic acids determined by their partitioning in the oil phase of an oil–water mixture (1:1, w/w) decreases in the following sequence (values in the parentheses give percent in the oil phase): FeA (49.6) ~ PG (49.4) > CoA (22.6) > CaA (0.30). The affinity of FeA for incorporation into microsomal membranes was reported to be 5% (*9*). In these works, the pH of the systems could affect both the partitioning pattern and the properties of the antioxidants. Nevertheless, on the basis of these reports, distribution of CaA, CoA, and FeA in the phospholipid bilayers at pH above the pK_a of the acidic group of the antioxidant molecules is expected to be lower than in the aqueous phase. Consequently, the radical scavenging abilities of these compounds are likely to be less involved in the inhibition of oxidation.

However, in free iron catalyzed oxidation a prooxidative effect was observed at 1 μM PG concentration (PG/Fe = 0.1). At low concentrations and in an abundance of iron, the capacity of PG to scavenge free radicals seems to be insufficient. Moreover, PG possesses a strong metal reducing power as verified in this study by the FRAP assay. The proportion of PG that is active as a free radical scavenger may be rapidly depleted and, at the same time, the proportion remaining in the aqueous phase may reduce ferric iron, resulting in an overall promotion of lipid oxidation.

Liposomes are charged particles (*13*). The charge of the particles may significantly affect the oxidation processes (*2*). The zeta potential, characterizing the electric potential difference between the particle surface and the surrounding aqueous phase, of cod roe phospholipid liposomes at pH 5.5 is strongly negative (~ −20 mV) (*14*). At pH 5.5, the molecules of CaA, FeA, and CoA exist mostly as anions because of the ionized acidic group [pK_a ~ 4.4 (*58*)]. Due to this, the accessibility of the negatively charged molecules toward the phospholipid interface may be hindered because of electrostatic repulsion into the aqueous phase by the negatively charged outer surface of liposomes. This may decrease the amount of molecules that can act as radical scavengers in the phospholipid interface and increase the proportion available for interactions with iron in the aqueous phase.

In additional experiments at pH 3.0, the prooxidative maximum of CaA was shifted to higher concentrations (~300 μM) and the maximum of oxidation was significantly lower compared to pH 5.5 (**Figure 4**), indicating the importance of pH. At pH below the pK_a value of the acidic group, the CaA molecules are mostly uncharged as the acidic group is protonated (*58*). Due to this, the polarity of the molecules and consequently the solubility of CaA in the water phase should decrease. Moreover, at pH 3.0 the zeta potential of liposomes is approximately zero, and a weaker catalytic activity of iron relative to pH 5.5 was observed in our earlier studies (*14*, *15*). A combination of these factors might facilitate a better accessibility of CaA molecules into the phospholipid bilayers, where they could act as radical scavengers. Both the shift in maximum and the overall lower degree of prooxidative activity at pH 3.0 could be attributed to these aspects because higher concentrations of CaA were needed to reach the maximum prooxidative effect.

A number of studies reported on the chelating properties of phenolics bearing catechol or pyrogallol moieties (*8*, *38*, *45*, *46*, *59*, *60*) as a mechanism that inactivates the redox cycling of metals by the formation of stable metal–antioxidant complexes (*1*). These studies are rather inconsistent regarding the degree of chelation; moreover, environmental conditions may also play a significant role.

Due to the strong pro-oxidative behavior of CaA, we observed that interactions between CaA and low molecular (free) iron are clear and apparently do not seem to produce any stable complexes which would reduce the concentration of free iron. In other words, the complex between iron and CaA would have reduced the concentration of free iron in solution, thereby shifting the

Table 3. Overview of the Results and Their Relative Comparison with Different Properties of the Studied Phenolics[a]

	inhibition of oxidation		antioxidant capacity assays				antioxidant properties[b]		
antioxidant (OH groups)	Fe	Hb	FC	FRAP	DPPH	ABTS	partitioning in oil[c](1)	chelating ability (2)	redox potential (3)
PG (3)	++++	++++	++++	++++	++++	++++	++++	++++	++++
CaA (2)	−	+++	++++	+++	+++	+	+	+++	+++
FeA (1)	++	++	++	++	++	+++	++++	−	++
CoA (1)	−	−	+	+	−	+++	++	−	+

[a] PG, propyl gallate; CaA, caffeic acid; FeA, ferulic acid; CoA, *p*-coumaric acid; + and − signs refer to the positivity or negativity/absence of the feature, respectively; the number of + signs refers to the strength of the feature within the group of studied phenolics, ++++ being the strongest. [b] Data based on literature findings: 1 = ref (*11*); 2 = ref (*11*, *59*, *60*); 3 = ref (*11*). [c] Partitioning in an oil phase of an oil−water (1:1) biphasic system.

equilibrium between free iron and iron bound to phospholipids, reducing the iron concentration on the lipid/water interphase, and finally reducing the rate of lipid oxidation. In this way, CaA would have shown an antioxidative effect. If there is such a complex binding between iron and CaA, the effect is clearly overshadowed by another reaction, intramolecular electron transfer, that increases the oxidation rate (**Figure 6**).

A schematic comparison between the experimental results and a correlation between the results and the antioxidants' properties that are discussed above is shown in **Table 3**. Good agreement was found between inhibition of Hb-catalyzed oxidation and the redox potential of the phenolics. The antioxidant capacity assays based on single electron transfer, that is, the FC and FRAP assays, also correlated well with the redox potentials. The findings of other laboratories on positive chelating ability of CaA toward iron were in great contrast to our observations in the iron-mediated oxidation.

In conclusion, the present study proves that the type of prooxidant, free iron versus hemoglobin, and the antioxidant-to-prooxidant ratio are factors of high importance for the efficiency of the studied phenolics in the inhibition of catalyzed oxidation of LC-PUFAs in liposomes. Therefore, when a particular phenolic compound is selected for an application in LC-PUFA-rich food emulsions, the type and content of different prooxidative agents should be known. Caffeic acid acted as a good antioxidant in Hb-promoted oxidation, whereas in free iron induced oxidation it was found to be a potent prooxidant, which indicates that different reactions are involved in interactions between free iron, Hb, and phenolic compounds. We assume reducing abilities of caffeic acid are responsible both for the promotion of free iron catalyzed oxidation and for the inhibition of Hb-catalyzed oxidation. Among the tested phenolics, only propyl gallate, a synthetic antioxidant, fulfilled the requirement for high efficacy both in free iron catalyzed and in Hb-catalyzed oxidation.

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PAPER III

The effect of dietary antioxidants on iron-mediated lipid peroxidation in marine emulsions studied by measurement of dissolved oxygen consumption

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Research Article

The effect of dietary antioxidants on iron-mediated lipid peroxidation in marine emulsions studied by measurement of dissolved oxygen consumption

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Addition of antioxidants into food containing fish omega-3 PUFA is an approach to protect the healthy omega-3 lipids from oxidation and to increase the oxidative stability of the food. Low molecular weight (LMW) iron (Fe^{2+}/Fe^{3+}) is a ubiquitous component in emulsified food and a mediator of lipid peroxidation even at trace levels. In this work, the effects of EDTA, citric acid, caffeic acid, propyl gallate, α-tocopherol, ascorbic acid, β-carotene, and astaxanthin on iron-mediated lipid peroxidation in 10% w/v herring oil emulsions stabilized with herring phospholipids (pH 3.5–5.5) were studied by measurement of the dissolved oxygen consumption by unsaturated fatty acids. EDTA and citric acid completely inhibited iron-mediated oxidation when they were added in twice the ratio to iron at pH>3.5. Caffeic acid, ascorbic acid, and α-tocopherol enhanced the oxidation by reducing Fe^{3+} to the more prooxidatively active Fe^{2+}, while propyl gallate reduced the oxidation by iron chelation. Ascorbic acid was depleted by interactions with iron and decreased the prooxidative activity of α-tocopherol. Astaxanthin and β-carotene showed minor effects on iron-mediated lipid oxidation. This study shows that the interactions between LMW iron and antioxidants have an impact on lipid peroxidation in emulsions in concentration dependent manners. Interactions with metals must therefore be considered when interpreting the activity/behavior of antioxidants in emulsions rich in omega-3 fatty acids. This study also shows that it is possible to screen these interactions by quantification of the dissolved oxygen consumption.

Practical applications: This work investigates the behavior of several food antioxidants in the presence of LMW iron in fish oil emulsions stabilized with marine phospholipids, and shows that interactions with iron can convert some of the anticipated antioxidants into pro-oxidants. Measurements of dissolved oxygen concentration have been used in this study to follow iron-mediated lipid peroxidation in the emulsions. It is shown that by quantification of the dissolved oxygen consumption it is possible to screen and evaluate the interactions between LMW iron and antioxidants in emulsion-type systems.

Keywords: Antioxidants / Emulsion / Iron / Marine lipids / Oxidation / Oxygen uptake

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Abbreviations: AV, anisidine value; **FAME,** fatty acid methyl ester(s); **LMW,** low molecular weight; **LOOH,** lipid hydroperoxide(s); **OUR,** oxygen uptake rate(s); **PL,** phospholipids; **PV,** peroxide value; **TBARS,** thiobarbituric acid reactive substances

1 Introduction

Incorporation of the healthy and physiologically important omega-3 lipids of marine origin into everyday food has become an increasing trend in the past 10 years. Typical examples of food fortified with omega-3 lipids are yoghurts, dairy and soft drinks, spreadable fats, and bread. One aspect of the fortification is to improve the insufficient intake of marine omega-3 fatty acids in modern societies and meet the recommended daily intake levels for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1]. Other aspects

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are to increase the nutritional and marketing value of the products. Marine lipids are susceptible to oxidative spoilage which complicates maintaining the original taste and odor, and the shelf-life of the fortified products [2]. In addition, oxidation of lipids produces reactive and toxic compounds which are believed to be harmful for the human body [3].

Low molecular weight (LMW) iron (Fe^{2+}/Fe^{3+}) is a potent mediator of lipid peroxidation even at trace (nanomolar) concentrations [4]. Degradation of enzymes, pigments, and metalloproteins during processing of raw materials, as well as ingredients, tap water (containing $\sim$200 μg Fe/L) and well/mineral water (containing >680 μg Fe/L) [5], steel processing equipment and packing materials, may deliver ionic iron into the food matrices. Therefore, LMW iron represents an important and ubiquitous element in food. LMW iron is found endogenously in food but may also be added exogenously to increase the nutritional value of the food. Products such as, yoghurts, milk, cheese, infant formulas, bakery products, cereals, and ingredients (salt, sugar, flour) may be fortified with up to 500 mg Fe/kg [6].

Addition of antioxidants to food is an approach to retard oxidation of sensitive marine lipids. Antioxidants can exhibit the antioxidant activity via a series of mechanisms, such as inhibition of radical oxygen species, quenching free lipid radicals, singlet oxygen, and photosensitizers, chelation of metals, inhibition of pro-oxidative enzymes, synergism with other antioxidants, and scavenging triplet oxygen [4].

Oil-in-water emulsions display similarities with liquid foods and have been widely used for investigation of the activity of both prooxidants and antioxidants, and the impact of environmental factors on oxidation of emulsified lipids [7–10]. The activity of antioxidants depends on their physical location in the emulsions and molecular structure, as well as on the chemical interactions with other compounds in the environment, the physical characteristics of the emulsion droplets and the overall system [11]. It is therefore desirable to understand the behavior of different antioxidants in variously complex emulsions to make it easier to develop oxidative stable products enriched with omega-3 fatty acids [12].

Sørensen et al. [8] reported that certain phenolic compounds in emulsions worked as prooxidants in the presence of iron, but they inhibited oxidation when the iron was not added. Osborn and Akoh [13] also reported prooxidant effects of some phenolic antioxidants in oxidation of structured lipid-based emulsions catalysed by iron. The same antioxidant added to different fish oil enriched food emulsions displayed different effects in the emulsions [12]. The knowledge on the influence of the ubiquitous iron on the activity of dietary antioxidants and the interactions between the iron and the antioxidants in emulsions may elucidate some of the variable behavior of antioxidants reported for the more complex emulsion-type systems.

Currently, there are not any fast and simple techniques for direct measurement of lipid oxidation in emulsions. To determine the oxidation status of the emulsified lipids, it is usually necessary to extract the oil phase from the emulsions before specific markers of lipid oxidation can be measured, or to evoke liberation of accumulated volatiles from the emulsion and determine the volatile content [14, 15]. An alternative approach is to monitor the concentration of the dissolved oxygen during lipid peroxidation, thus focusing on the loss of an essential lipid oxidation substrate. Mozuraityte et al. [16] showed that a decrease in the dissolved oxygen consumption was proportional to the increase of concentration of lipid peroxides and thiobarbituric acid reactive substance (TBARS) during oxidation of cod roe phospholipids in liposomes mediated by free iron. Therefore, oxygen uptake reflects the rate of lipid oxidation, if side reactions consuming oxygen are eliminated or not present.

The reaction mechanisms and the impact of a series of factors on LMW iron-mediated lipid peroxidation in marine emulsions and liposomes have been studied by means of the dissolved oxygen consumption by Mozuraityte et al. [16] and Kristinova et al. [7]. The present paper utilizes the oxygen uptake method to study the influence of several food antioxidants, namely ethylenediaminetetraacetic acid (EDTA), citric acid, caffeic acid, propyl gallate, α-tocopherol, ascorbic acid, β-carotene, astaxanthin, and combinations of α-tocopherol with ascorbic acid and EDTA, on LMW iron-mediated lipid peroxidation in 10% herring oil emulsions (pH 3.5–5.5) stabilized with herring phospholipids, and characterizes the mutual interactions between iron and antioxidants.

2 Materials and methods

2.1 Materials

Mature roe from Norwegian spring spawning herring (*Clupea harengus*), obtained from Grøntvedt Pelagic (Uthaug, Norway), was used for isolation of marine phospholipids. Before isolation the intact roe glands were kept frozen at −40°C. Herring (*Clupea harengus*) oil was produced by SINTEF Mobile Plant from an assortment of ultra-fresh herring rest raw material, consisting of heads, guts, and muscle trimmings including bones and skin, obtained from Grøntvedt Pelagic (Uthaug, Norway). Thermal processing (70°C) of the rest raw material was used to separate the oil. More information on the production of crude herring oil is available in the work of Carvajal [17]. The crude herring oil was kept at −30°C until further processing.

2.2 Chemicals and reagents

All chemicals and solvents used in this study were of analytical or synthetic grade, and were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany), Merck KGaA (Darmstadt, Germany), or Fluka Chemie (Buchs, Germany). Nitrogen (99.99% N_2), hydrogen (99.99% H_2), and helium gas (99.99% He) were provided by AGA AS, Oslo. Analytical standards of FAME and lipid classes standard mixes were

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purchased at Nu-Check Prep, Inc. (Elysian, MN, USA). Phospholipid standards were purchased at Avanti Polar Lipids, Inc. (Alabama, USA), and standards of individual lipid classes were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Solvents used in TLC-FID, GC-FID, and HPLC-CAD analyses were of chromatography grade. Distilled water was used for preparing aqueous solutions, and deionised water (0.056 μS/cm) was used in TLC-FID, GC-FID, and HPLC-CAD analyses.

2.3 Polishing of crude herring oil

To remove impurities in the crude oil, 10% w/w of boiling water (in relation to the oil quantity) was added to the oil and the liquid was stirred manually for 10 min. The warm mixture was centrifuged (7000 rpm for 10 min at 40°C) and the clear oil phase was collected, divided into ~25 mL portions, and kept at −20°C in closed plastic flasks in darkness until needed [18]. The polishing step was expected to reduce impurities commonly present in crude oils, such as proteins, free fatty acids, phospholipids, minerals (including pro-oxidative metals), antioxidants, pigments, sterols, and insoluble particles [19].

2.4 Isolation of phospholipids

The frozen roe glands were allowed to thaw overnight at 4°C. The extraction of total lipids from the roe was performed according to the method of Bligh and Dyer [20]. The phospholipids were isolated from the total lipids by precipitation in cold (−20°C) acetone, as initially described by Kates [21] and modified by Mozuraityte et al. [22]. The isolated phospholipids dissolved in chloroform were stored at −20°C in closed flasks. An aliquot necessary for analyses/experiments was pipetted out when needed.

2.5 Peroxide value

The level of lipid hydroperoxides in the polished herring oil was assessed by determination of peroxide value (PV) employing iodometric titration with potentiometric titration end-point determination, as described by Kristinova et al. [7]. The maximum coefficient of variation for this method was found 9.1% and the limit of quantification (LOQ) was found 1.1 mmol LOOH/kg. Due to a limited quantity, PV in the isolated phospholipids was analyzed by a spectrophotometric ferro-thiocyanate assay described with modifications by Kristinova et al. [7]. The maximum coefficient of variation for this method was found 22.7% and the LOQ was found 2 mmol LOOH/kg. Three to five replicates were analyzed and the results are expressed as a mean value ± SD.

2.6 *p*-Anisidine value

p-Anisidine value (AV) in the lipids was determined according to the AOCS Official Method [23] using an Ultrospec 2000 UV/VIS spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Three replicates were analyzed and the results are expressed as a mean value ± SD.

2.7 Thiobarbituric acid reactive substances

The concentration of TBARS in the lipids was determined by a spectrophotometric method described by Ke and Woyewoda [24]. All amounts were reduced to one half relative to the given procedure. The analysis was performed with three parallels for the oil and five parallels for the PL. The results were expressed in mmol TBARS/kg lipids as a mean value ± SD.

2.8 Total carotenoids

The content of carotenoids (mainly astaxanthin) in the lipids was determined spectrophotometrically [25]. Briefly, the lipids were dissolved in *n*-hexane to a known concentration, and the absorbance of the solution was measured at 472 nm against pure *n*-hexane using an Ultrospec 2000 UV/VIS spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). The carotenoid content was calculated using a standard absorbance for all-*E*-astaxanthin, $E = 2100$ $(g/100\,mL)^{-1}$ cm^{-1} [standard absorbance of 1% v/w astaxanthin solution in a cuvette with an optical path 1 cm in *n*-hexane at 470 nm]. The samples were analyzed in triplicate and the results are expressed in mg carotenoids/kg lipids as a mean value ± SD.

2.9 Lipid classes

Lipid classes in the lipids were analyzed by a TLC with FID system (Iatroscan TLC-FID analyzer MK-6, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). The procedure described by Fraser et al. [26] was followed.

2.10 Fatty acid profile

Methylation of fatty acids (FA) was performed as follows: 10 mg of oil in a stoppered glass centrifuge tube were dissolved in chloroform containing 10% (in relation to the mass of oil) of internal standard (heneicosanoic acid, 21:0). Chloroform was completely evaporated by N_2 gas and the oil was redissolved in 1 mL of 0.5 M NaOH in methanol. The lipids were hydrolyzed for 15 min at 100°C, and cooled. Two milliliters of 10% BF_3 in methanol was added and the mixture was incubated for 5 min at 100°C, and cooled. Afterwards, 1 mL of hexane was added and the mixture was incubated for 1 min at 100°C, and cooled. Finally, 0.5 mL of hexane and 2 mL of saturated NaCl solution was added, the mixture was vortexed and centrifuged at 2000 rpm for 3 min. The hexane phase containing FAME was collected, diluted with 0.5 mL hexane, and centrifuged again. The latter step was repeated once more with the collected hexane phase.

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Fatty acid composition in the methylated samples was analyzed by an Agilent Technologies 7890A gas chromatograph with flame ionization detection (GC-FID) system equipped with 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA) according to Dauksas et al. [27] with the following modifications: The detector temperature was held at 270°C, and the flame was maintained with 25 mL/min H_2 gas and 400 mL/min filtered air. Chromatography was carried out using a Cp-wax 52CB, 25 m × 0.25 mm with id = 0.2 μm column (part no. CP7713, Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The GC inlets were held at 250°C. The initial oven temperature was 80°C and it was increased to 180°C at 25°C/min with a 2 min hold, followed by an increase to 205°C at 2.5°C/min with a 6 min hold, followed by a final increase to 215°C at 2.5°C/min with a 4 min hold. Fatty acids were identified by comparison to the retention times of commercial standards and quantified by the internal standard as FA/g oil. The accuracy of the method was verified by comparison of FA profiles of selected marine oils against profiles assessed by accredited laboratories. The results are expressed as average % of each FA to a total FA amount of two replicates with a maximum coefficient of variation (CV) 9.6%. Quantities <0.23% were uncertain.

2.11 Phospholipid classes

The phospholipid classes in the isolated PL were analyzed by the Agilent 1260 Infinity HPLC system (Agilent Technologies, Germany) coupled to the Corona Ultra Charged Aerosol Detector (CAD) (ESA/Thermo Scientific/Dionex, USA). The PL were dissolved in isopropanol (1 mg/mL) and separated on Agilent Prep-SIL Scalar 10 μm column, 4.6 × 150 mm (packed by Agilent Technologies, Santa Clara, CA, USA) kept at a constant temperature (22.0 ± 0.8°C). For the isocratic elution a ternary gradient having a constant flow rate of 1.25 mL/min and consisting of A = *n*-hexane, B = 2-propanol, and C = deionized water was used with the following timetable: at 0.00 min 40:59:1 (%A/%B/%C); at 3 min 40:54:6; at 18.00 min 40:50:10; at 18.01 min 40:59:1; and at 23 min 40:59:1. The sample temperature was 4°C and the injected volume was 10 μL. Retention times of PL standards were used for peak identification and standard curves of the same standards were used for quantification of the PL classes. Duplicate analysis was performed and the results are average values with maximum coefficient of variation 3.5%.

2.12 Preparation of emulsions

A PL-oil mixture was prepared first by mixing an aliquot of the oil with an aliquot of the PL dissolved in chloroform so that the net PL formed 9% w/w in the oil. Mixing was performed in a round bottom flask attached to a vacuum rotavapor during simultaneous evaporation of the chloroform from the mixture by a rotavapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) (1 h, 30°C, 30 mbar). The mixture was kept at −20°C.

Ten percent w/v oil-in-water emulsions stabilized with PL were prepared by emulsifying distilled water with polished herring oil pre-mixed with herring roe PL (9% w/w lipid base). The emulsification was performed with an Ultra Turrax T10 Basic Disperser with 10 mm (diameter) blade (Janke & Kunkel, IKA, Staufen, Germany). The water was poured to the lipids during the first 5 s of emulsification, and emulsification time of 30 s with gradual increase of the blade rotation from 8000 to 30 000 rpm was applied. These conditions gave a stable and also the narrowest droplet size distribution (published in [7]).

2.13 Droplet size

The droplet size distribution was determined using a Mastersizer 3000 (Malvern Instruments Ltd., UK). Briefly, a few drops of the emulsion were added to a circulating water bath until an obscuration of 6–12% was reached. The emulsion was gently shaken before the drops were taken to eliminate the influence of creaming and flocculation. The refractive indices of cod liver oil (1.481) and water (1.330) were used for particle and dispersant index, respectively. The average droplet diameter (volume based, Dv50) is expressed in μm as a mean value ± standard error (SE) of five replicates.

2.14 Zeta potential

The zeta potential of emulsion droplets (at pH 5.5) was determined from electrophoretic mobility measurements at 25°C using a Zetasizer Nano ZS (Malvern Instrument Ltd., UK). The emulsions were prepared in three replicates, each replicate was measured three times consecutively, and the results were pooled. The pooled result is expressed in mV as a mean value ± SD.

2.15 Preparation of pro- and antioxidant solutions

Stock solutions of Fe^{2+} and Fe^{3+} (20 mM) were prepared by dissolution of $FeSO_4 \cdot 7H_2O$ and $FeCl_3$, respectively, in 0.5 M HCl to minimize iron precipitation. Working solutions of Fe^{2+} and Fe^{3+} were prepared daily by diluting an aliquot of the stock solutions with distilled water to a desired concentration. For addition of different concentrations of iron into the emulsion, the volume of the work solution was kept constant (10 μL) while the concentration of the working solution was varied.

Stock solutions (100 mM) of propyl gallate (PG), ascorbic acid (AsA), and caffeic acid (CaA) were prepared in 96% ethanol due to a limited solubility in water. Citric acid (CA) and EDTA were dissolved in distilled water to a concentration of 50 and 0.75 mM, respectively. Working solutions (2.5–20 mM) were prepared daily by dilution of the stock

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solutions with distilled water with the exception of EDTA for which the stock solution was used directly.

Stock solutions of α-tocopherol, β-carotene, and astaxanthin were prepared in chloroform to a concentration of 5 mM. To obtain a desired concentration and a thorough immixture of the antioxidant in the emulsion, specific aliquots of the stock solution were added to the oil-emulsifier mixture prior to evaporation of the chloroform and emulsion preparation (described in Section 2.12).

2.16 Oxygen uptake measurements

Oxidation of fatty acids in the emulsions was followed by measuring dissolved oxygen consumption using the Oxygraph system (Hansatech Instruments Ltd., Norfolk, UK) as described in earlier studies by the authors [7, 28]. Briefly, 1 mL of emulsion was transferred into an oxygraphic cell surrounded by a water jacket maintaining a constant temperature. The concentration of the dissolved oxygen was measured by a Clark polarographic oxygen electrode which formed the floor of the reaction cell. The electrode was calibrated with oxygen saturated and oxygen depleted distilled water; oxygen was depleted by adding $Na_2S_2O_4$. The cell was equipped with a magnetic stirrer and closed with a plunger with a capillary opening preventing access of air oxygen and at the same time allowing injection of solutions. The concentration of the dissolved oxygen (μM) was continuously recorded as a function of time (min), giving continuous oxygen concentration curves. In order to re-establish saturation conditions, infusion of air was performed when the concentration of the dissolved oxygen in the cell reached almost zero. As a measure of oxidation, oxygen uptake rates (OUR) were calculated from the recorded curves. Three cells were run simultaneously for each experiment and the OUR were expressed in μM O_2/min as a mean value ± SD.

2.17 Oxidation experiments

All the oxidation experiments were performed at 30°C. The pH of freshly made emulsions varied between 5 and 6 (average pH = 5.6 ± 0.4 (SD), n = 82). The pH in the emulsions was re-measured after each oxidation experiment (pH of experiment), because addition of the antioxidant solutions and the acidic iron solutions lowered the original pH to pH 3.5–4.5. The concentrations and combinations of pro- and antioxidants that were investigated are listed in Table 1; the chemical structures of the antioxidants are given in Fig. 1.

The calculations of the inhibitory effects of the antioxidants in the emulsions are schematically shown on a representative oxygen consumption curve depicting the addition of an antioxidant and thereafter a prooxidant (Fe^{2+}, Fig. 2). The background oxygen consumption (R_0) was recorded for 5–10 min before addition of the antioxidant solution. Afterwards, a secondary background oxygen consumption (R_B) was recorded for 5–10 min before addition of the Fe^{2+} working solution. The oxygen consumption after the iron addition was recorded for 10–30 min and the total OUR was quantified (R_T). The net OUR was found by subtracting R_B from R_T. In control experiments (no antioxidants), a blank antioxidant solution was added instead, and the calculations were done analogically. In emulsions containing α-tocopherol, astaxanthin or β-carotene, R_B was recorded from the start. Immediately after addition of Fe^{2+}, a drop in the oxygen concentration (Δ O_2) occurs. This drop is referred to as "the equilibrium drop" in the text (more details in Section 3.2). The magnitude of the drop and the inhibition

Table 1. Concentrations and combinations of Fe^{2+}/Fe^{3+} and antioxidants added to the emulsions

Pro-oxidant		Antioxidant	
Iron	Concentration (μM)	Compound	concentration (μM)
Fe^{2+}	25	–	–
Fe^{3+}	25	–	–
Fe^{2+}	25	EDTA	7.5, 15, 25, 50
Fe^{3+}	25	EDTA	25
Fe^{2+}	25	Citric acid	7.5, 12.5, 25, 50
Fe^{3+}	25	Citric acid	25
Fe^{2+}	25	Caffeic acid	10, 25, 50, 100, 200, 500
Fe^{3+}	25	Caffeic acid	25
Fe^{2+}	25	Propyl gallate	25, 100, 200, 500
Fe^{3+}	25	Propyl gallate	100
Fe^{2+}	25	Ascorbic acid	25, 50, 100, 200
Fe^{2+}	25	α-Tocopherol	100, 200, 300
Fe^{2+}	25	β-Carotene	100, 200, 300
Fe^{2+}	25	Astaxanthin	100, 200, 300
Fe^{3+}	25	Astaxanthin	100, 200, 300
Fe^{2+}	25	Ascorbic acid + EDTA	50 50
Fe^{2+}	25	α-Tocopherol + Ascorbic acid	100 50, 100, 200
Fe^{2+}	25	α-Tocopherol + Ascorbic acid	100, 200, 300 50

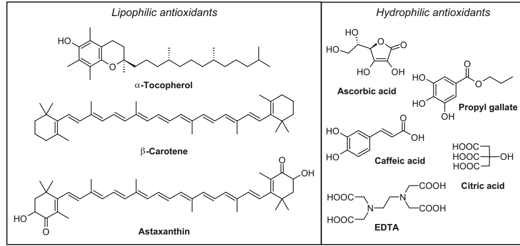

Figure 1. Chemical structures of antioxidants in this study.

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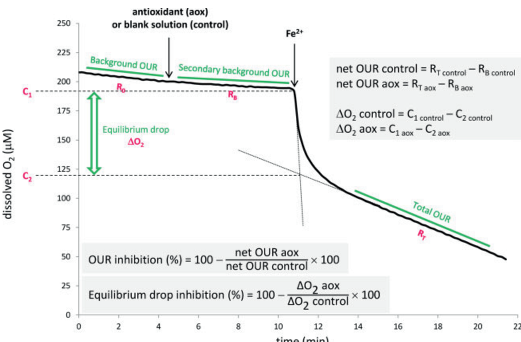

Figure 2. A representative oxygen concentration curve in emulsion with addition of an antioxidant and thereafter a prooxidant (Fe^{2+}). The figure illustrates the description of the OUR and the equlibrium drop in oxygen concentration after addition of Fe^{2+}.

of the drop by antioxidants were quantified as well (Fig. 2). Oxyg32 software was used for the recordings of the oxygen consumption curves and quantifying the OUR and the magnitudes of the equilibrium drops.

The carriers for propyl gallate, ascorbic acid, and caffeic acid were water–ethanol solutions. Injecting 96% ethanol or distilled water (50 μL) to the emulsion had no or only marginal influence on the background OUR and on iron-mediated lipid oxidation in the emulsions (data not shown).

2.18 pH determination

The pH of the solutions and emulsions was measured by a TIM900 Titrator manager (TitraLab, Radiometer Analytical ASA, Copenhagen, Denmark) coupled with a combination glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, USA), which was calibrated daily against standard buffer solutions, pH 4.0 and 7.0, at 22°C.

2.19 Statistical analyses

Microsoft Excel 2010 was used for calculations and data processing. A statistical program Minitab® (version 16.2.3) was used for statistical analyses. To assess significant differences, the data were subjected to analysis of variance (one-way ANOVA), followed by a Tukey test. The level of significance was set to 95% ($p = 0.05$).

3 Results and discussion

3.1 Characterization of lipid substrates and emulsion

The oxidation status characterized by PV, AV and TBARS, the carotenoid content, fatty acid composition and profile of lipid classes in the lipids used for preparation of the emulsions are given in Table 2.

Based on the lipid hydroperoxide (LOOH) content determined in the individual lipid substrates, the 10% oil-in-water emulsions with phospholipids (PL) as emulsifier theoretically contained 10.2 mmol LOOH/kg lipid base, out of which 3.4 mmol LOOH/kg were in the PL fraction (emulsifier), and 6.8 mmol/kg were in the core of the emulsion droplets, i.e., in the TAG fraction. The values (lipid base) for AV, TBARS and total carotenoids were calculated analogically and are given in Table 2.

The pre-existing LOOH in the emulsion, especially in the phospholipid interphase, are of crucial importance for iron-mediated lipid oxidation [7], and the endogenous carotenoids may affect the rate of lipid peroxidation due to the ability of astaxanthin to scavenge lipid free radicals [29, 30]. Prior to emulsification, the PL and TAG were thoroughly blended under vacuum in a desired ratio. The subsequent emulsification, which was carried out at air atmosphere, might have aided a partial decomposition of the pre-existing LOOH and/or more likely a formation of new LOOH. Therefore, the calculated PV in the PL and TAG fractions may represent the minimum levels in the freshly made emulsions.

During the preparation of the emulsion, a depletion of the endogenous astaxanthin in both the PL and TAG phase may have occurred. In addition, astaxanthin might have become evenly distributed in the PL-TAG mixture and consequently also in the emulsion droplet, which would significantly decrease the astaxanthin content in the PL interface. Therefore, the total calculated value for carotenoids (2.6 mg/kg lipid base) may reflect the maximum level in both fractions.

The crude herring oil was polished in order to remove impurities. More details on the effect of the polishing step on the herring oil characteristics are available in Kristinova et al. [7]. The paper also reports the influence of the emulsification time on the particle size distribution and physical stability. The average droplet size (volume based) in the emulsions was $Dv50 = 10.9 \pm 0.1$ (SE) μm ($n = 25$) and the zeta potential of the droplets reflecting the surface charge, was -13 ± 3 (SD) mV ($n = 5$) at pH 5.5 ± 0.2.

The fatty acid profile of the PL and TAG revealed large differences in the proportions of mono-, di-, and poly-unsaturated fatty acids (double bond ≥ 3, $C \geq 18$) and the ratio between EPA and DHA (Table 2). Because the PL and TAG form two distinct phases in the lipid droplet, i.e., the interphase and the core of the droplet, the fatty acid profiles determined for PL and TAG unequivocally characterize the profiles in the interphase and the core of the droplet, respectively.

3.2 Iron-catalyzed lipid oxidation in emulsions

Lipid oxidation in the 10% herring oil emulsion was followed by a continuous recording of the dissolved oxygen

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Table 2. Oxidation status characterized by PV, AV and TBARS, carotenoid content and lilpid composition of the isolated herring roe phospholipids and the polished herring oil

Characteristics (lipid base)	Herring roe phospholipids (PL)	Polished herring oil (TAG)	10% emulsion PL + TAG = total[a)]
PV (mmol LOOH/kg)	37.6 ± 1.1	7.47 ± 0.09	3.4 + 6.8 = 10.2
AV	16.5 ± 0.8	1.61 ± 0.12	1.5 + 1.5 = 3.0
TBARS (mmol/kg)	1.1 ± 0.3	0.47 ± 0.03	0.1 + 0.4 = 0.5
Total carotenoids (mg/kg)	10.8 ± 0.7	1.80 ± 0.09	1.0 + 1.6 = 2.6
Lipid classes (%)			Not calculated
TAG	<LOD	98.9	
Cholesterol	<0.8	<0.8	
Unspecified	<0.8	<0.8	
PL, of which	99.5	<0.8	
PC	84.4	NA	
lysoPC	0.5	NA	
PE	14.1	NA	
lysoPE	0.6	NA	
Unspecified	0.3	NA	
Fatty acid profile (%)			Not calculated
Saturated	27.8	22.4	
Mono-unsaturated of which	16.7	59.1	
CET	2.9	38.1	
ERU	0.5	2.2	
GAD	9.2	25.8	
OLE	35.2	19.7	
Di-unsaturated[b)]	1.0	1.7	
Poly-unsaturated (db ≥ 3) of which	54.5	16.8	
EPA	23.4	40.5	
DHA	69.0	40.0	
DPA	2.0	4.3	

[a)]Theoretical values calculated from the values determined for oil (TAG) and phospholipids (PL), the total value is a summation of the contributions from PL and TAG;
[b)]Predominant di-unsaturated fatty acid was linoleic acid (C18:2 *n*6, all-cis).
NA, not analyzed; LOD, limit of detection; db, double bond; the values are given as a mean value ± standard error or SD or with a coefficient of variation (specified in Section 2 under each analysis); CET, cetoleic acid; ERU, erucic acid; GAD, gadoleic acid; OLE, oleic acid; DPA, docosapentaenoic acid.

consumption by fatty acids. Iron ions, ferrous (Fe^{2+}) and ferric (Fe^{3+}), were added to the emulsion (25 μM) to catalyze lipid oxidation. The concentration of iron added to the emulsions is relevant for food rich or enriched with iron [6]. Iron-mediated oxidation and the oxygen uptake measurements in marine emulsions and liposomes have been thoroughly described in the earlier papers of the authors [7, 16], therefore only the key aspects will be repeated here for the convenience of the reader.

The addition of Fe^{2+} to the emulsion resulted in a rapid drop in the dissolved oxygen concentration immediately after the addition (this phenomenon is referred to as "the equilibrium drop") followed by a linear decrease in the oxygen concentration (illustrated in Fig. 2). When Fe^{3+} was added, no drop occurred and only the linear decrease in oxygen concentration was observed (not shown). Since the magnitude of the drop can give additional information on interactions between the LMW iron and antioxidants, most experiments in this study were performed using Fe^{2+}.

The drop in Fe^{2+}-mediated oxidation occurs due to establishment of concentration equilibrium between Fe^{2+} and Fe^{3+}. The equilibrium is established via rapid oxidation of Fe^{2+} to Fe^{3+} by pre-formed LOOH. Fe^{2+} decomposes LOOH by a rate which is several orders of magnitude higher than the rate for Fe^{3+} [4, 31, 32]. This rapid decomposition of LOOH generates alkoxyl radicals (LO·) which rapidly abstract hydrogen from adjacent fatty acids producing lipid alkyl radicals (L·) which then rapidly react with the dissolved oxygen. This rapid cascade of reactions is manifested as the drop in the dissolved oxygen concentration after Fe^{2+} addition. The linear decrease in the OUR takes place once the equilibrium between Fe^{2+} and Fe^{3+} is established and a slower reaction between Fe^{3+} and LOOH becomes rate limiting. As a consequence, the concentration ratio between

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Fe^{2+} and Fe^{3+} becomes equal regardless of the original ions, and each iron atom oscillates between the oxidized and reduced form. Phosphate groups within the phospholipid heads (PL-phosphates) were proposed to be the binding sites for iron ions in the phospholipid interfaces in emulsions stabilised by PL and liposomes [7].

3.3 Chelators: Citric acid and EDTA

The effect of different concentrations (0–50 μM) of two chelators, citric acid and EDTA, on the OUR and the equilibrium drop in Fe^{2+}-mediated oxidation (25 μM) is shown in Fig. 3A.

Both EDTA and citric acid reduced the OUR in iron-mediated oxidation as a result of iron chelation, but only EDTA reduced the equilibrium drop. Increased concentrations of the chelators lead to a greater inhibition until the molar chelator-to-Fe^{2+} ratio was 2:1 at which a complete inhibition was achieved. At molar ratios $\leq$ 1:1 (excess of iron) citric acid inhibited the oxidation rates to a larger extent than EDTA.

The pH of the environment affects the metal binding abilities of chelators, since the COOH groups need to be partially or fully deprotonated in order to be able to chelate metals [32]. The pH in the emulsions after addition of the acidic iron solution dropped to 3.5 ± 0.5. At this pH one out of three OH^- groups in the citric acid molecule ($pK_1 = 3.12$, $pK_2 = 4.76$, $pK_3 = 6.41$ [33]), and two out of four OH^- groups in the EDTA molecule ($pK_1 = 1.99$, $pK_2 = 2.67$, $pK_3 = 6.16$, and $pK_4 = 10.26$ [34]) are dissociated.

Citric acid did not have any impact on the equilibrium drop at the tested Fe^{2+} concentrations which indicates that citric acid did not form a complex with Fe^{2+}. Francis and Dodge [35] reported that at pH 3.5 citric acid formed a tridentate complex with Fe^{3+}, $[Fe^{3+}(OH)Cit]^-$, while a tridentate complex between Fe^{2+} and citric acid, $[Fe^{2+}Cit]^-$, was not formed until pH 5. Since the pH of the emulsion was around 3.5, this may explain why citric acid was not able to reduce the equilibrium drop. Nevertheless, inhibition of OUR initiated by Fe^{2+} was still observed, presumably due to binding of Fe^{3+} formed during the red-ox cycling of iron. Citric acid has an ability to bind more than one Fe^{3+} ion [36], which may explain why citric acid was more efficient than EDTA when iron was in molar excess.

Increasing concentrations of EDTA resulted in reduction of the equilibrium drop demonstrating the ability of EDTA to bind Fe^{2+}. The inhibitory effect of EDTA was greatly improved when the concentration of EDTA was higher than that of iron, which is similar to the results found by Hu et al. [37].

The inhibitory effects of citric acid and EDTA on Fe^{2+}- and Fe^{3+}-mediated oxidation at the chelator-to-iron molar ratio 1:1 were compared (Fig. 3A). Once the equilibrium between Fe^{2+} and Fe^{3+} is established, an equal effect of each chelator on both Fe^{2+} and Fe^{3+} was expected, but not observed. Fe^{3+}-mediated oxidation was inhibited completely by both EDTA and citric acid, while Fe^{2+}-mediated oxidation was inhibited by 78 and 92% by EDTA and citric acid, respectively.

Kristinova et al. [7] suggested that iron ions are associated with the phosphate groups within the phospholipid heads (PL-phosphates) in PL stabilized emulsions and liposomes, which facilitates contact with hydroperoxide groups on fatty acids. A competition between EDTA or citric acid and the PL-phosphates for binding of iron may have an impact on the chelation efficacy of the two chelators. Nevertheless, the total inhibition of OUR at the excess of the chelators demonstrates the efficiency of both EDTA and citric acid to retrieve the iron ions from the droplet interface.

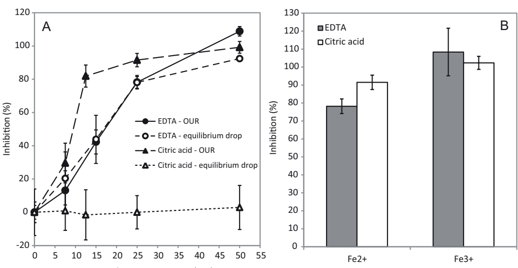

Figure 3. (A) Inhibition (%) of the oxygen uptake rate (OUR) and the equilibrium drop by different concentrations of EDTA and citric acid in Fe^{2+} (25 μM)-mediated lipid oxidation; (B) inhibition effects (%) of EDTA and citric acid on Fe^{2+}- and Fe^{3+}-mediated lipid oxidation at the chelator-to-iron ratio 1:1 for concentrations 25 μM. The results are the mean values ± SD ($n = 3$).

3.4 Caffeic acid

The effect of different concentrations of caffeic acid (10–500 μM) on Fe^{2+}-mediated oxidation (25 μM) was studied. Addition of caffeic acid resulted in a significant increase in the OUR; in other words, caffeic acid behaved as a pro-oxidant. The increase (%) in the net OUR and the reduction (%) of the equilibrium drop as a function of caffeic acid concentration are plotted in Fig. 4.

Caffeic acid enhanced the OUR over the whole tested concentration range with a maximum prooxidative effect at a caffeic acid-to-iron molar ratio = 1:1 (25 μM). The OUR decreased with the excess of caffeic acid, but even the highest concentration (500 μM, caffeic acid-to-iron ratio = 20:1) did not suppress the prooxidative behavior. The prooxidative behavior of caffeic acid was also observed in oil-in-water emulsions [8] and in liposomes [38], where the prooxidative effect was explained by the ability of caffeic acid to reduce Fe^{3+} to the more catalytically active Fe^{2+}. In the latter study, a maximum prooxidative activity of caffeic acid at pH 3.0 was observed at a caffeic acid-to-iron ratio about 20:1. This might

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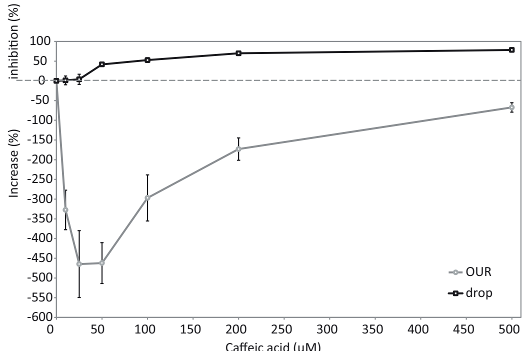

Figure 4. Increase (%) in the net OUR (circles) and reduction (%) of the equilibrium drop (squares) at different concentrations of caffeic acid when Fe^{2+} (25 μM) was used as a prooxidant. The results are the mean values ± SD ($n=3$).

be attributed to structural and compositional differences between emulsion droplets and liposome vesicles – e.g., the total surface area, which is 100-fold larger for emulsions than for liposomes, and different fatty acids composition of the phospholipids forming the interphase [7].

The decreasing prooxidative effect of caffeic acid at the caffeic acid-to-iron ratios greater than 1:1 (excess of caffeic acid) could be attributed to the radical scavenging abilities of caffeic acid [39]. To function as a chain-breaking antioxidant caffeic acid needs to be in the vicinity of the lipid phase. Caffeic acid has a polar character which could allow its location or association with the phospholipid interface, specifically the phospholipid heads of the emulsions droplets, which are in contact with the surrounding aqueous phase.

Alternatively, caffeic acid could chelate iron ions to some degree. Several papers reported chelating abilities of phenolic acids [40–42]. The binding constant for caffeic acid-Fe complexes was however reported to be much lower ($8.12\,M^{-1}$) in comparison to strong chelators, such as EDTA ($4.9\times10^{8}\,M^{-1}$), indicating that caffeic acid is a weak chelator. Even at the highest caffeic acid concentration (500 μM) the prooxidant effect was not suppressed, showing that reduction of iron is still prevailing at these conditions.

Caffeic acid reduced the amount of oxygen consumed within the equlibrium drop when it was in excess to iron – the higher the concentration of caffeic acid, the higher the reduction of the drop, although this effect was not proportional. The reduction in the equilibrium drop could be a consequence of a partial iron chelation combined with radical scavenging, as mentioned above.

The effect of caffeic acid (25 μM) on Fe^{2+}-mediated oxidation was compared to oxidation mediated by Fe^{3+} (both iron ions at 25 μM). The OUR were somewhat higher when the oxidation was induced by Fe^{2+} (43 ± 7 μM O_2/min) compared to Fe^{3+} (34 ± 3 μM O_2/min), but the values were not significantly different. Once caffeic acid is in excess to iron, there seems to be a competition between iron reduction, scavenging lipid radicals and metal chelation. This may result in decreased pro-oxidative activity with increasing caffeic acid concentration in relation to iron. Eventually the anti-oxidative abilities may prevail. In this respect, the final behavior of caffeic acid is both caffeic acid and iron concentration dependent. This could at least partially explain different effects of caffeic acid reported in various studies [43].

3.5 Propyl gallate

The effect of different concentrations of propyl gallate (10–500 μM) on the net OUR and the equilibrium drop in Fe^{2+} (25 μM)-mediated oxidation is shown in Fig. 5. All the tested concentrations inhibited both the net OUR and the drop. The degree of inhibition increased with increasing concentration of propyl gallate, although complete inhibition was not obtained.

The antioxidant activity of propyl gallate is related to its ability to donate hydrogen to lipid radicals, thus terminating the propagation of lipid oxidation. Propyl gallate may scavenge lipid radicals formed during the reaction between Fe^{2+} and pre-formed LOOH, i.e., during the equilibrium drop, thus eliminating the radicals which would otherwise react with oxygen. As a consequence both the equilibrium drop and the OUR are reduced. The less polar character of propyl gallate enables it to up-concentrate at the interface in the emulsion where lipid oxidation primarily takes place. Propyl gallate also has the ability to chelate iron at acidic pH, which could also explain the antioxidant effect [42].

The net OUR were significantly lower when the oxidation was induced by Fe^{3+} (1.9 ± 0.6 μmol O_2/min) than Fe^{2+} (5.30 ± 0.11 μmol O_2/min) in emulsions containing propyl

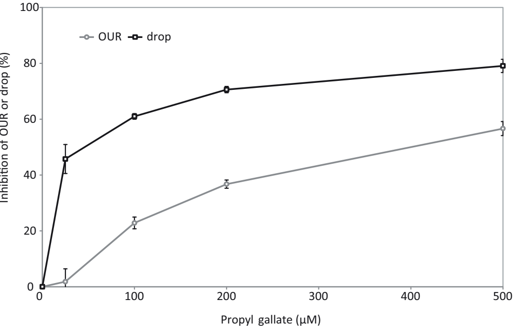

Figure 5. Inhibition (%) of the net OUR (circles) and the equilibrium drop (squares) at different concentrations of propyl gallate when Fe^{2+} (25 μM) was used as a prooxidant. The results are the mean values ± SD ($n=3$).

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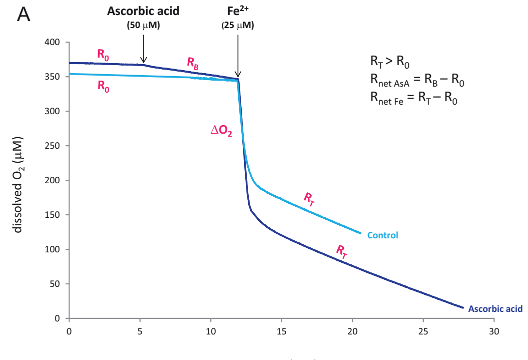
gallate (100 μM), which indicates that propyl gallate was more efficient regarding the inhibition of Fe^{3+}-catalyzed oxidation. In the study by Kristinova et al. [38], no difference between the ability of propyl gallate to inhibit Fe^{2+}- and Fe^{3+}-catalyzed lipid oxidation in liposomes was observed. It is not clear at the moment why the oxidation induced by Fe^{3+} was inhibited better in the emulsions. It could be argued that chelation/retrieval of Fe^{3+} by propyl gallate from the phospholipid interphase can be more efficient than of Fe^{2+} under the given conditions.

3.6 Ascorbic acid

The addition of Fe^{2+} (25 μM) to emulsions containing ascorbic acid (25–200 μM) resulted in large equilibrium drops, which were larger than for the control, i.e., Fe^{2+}-mediated oxidation without any antioxidant. The magnitude of the drops dramatically increased with increasing concentration of ascorbic acid (e.g., 25 μM ascorbic acid is shown in Fig. 6A) which made quantification of the drop as well as the following slower oxygen consumption in many cases impossible, because the oxygen was rapidly and entirely depleted in the emulsion.

In aqueous systems, ascorbic acid is known to interact with LMW transition metals dissolved in the aqueous phase [44, 45]. Reduced metals (Fe^{2+}) aid ascorbic acid oxidation through the formation of a ternary complex of ascorbate monoanion-O_2-Fe^{2+}, yielding dehydroascorbic acid and H_2O. This mechanism is accompanied by oxygen consumption and can prevent lipid oxidation due to removal of dissolved triplet oxygen from the system. Oxidized metals (Fe^{3+}) can be converted by ascorbic acid into the reduced states (Fe^{2+}), yielding dehydroascobic acid as well. This mechanism is not accompanied by oxygen consumption, but yields reduced metals which are more active in lipid oxidation. In the latter mechanism ascorbic acid behaves as a reductant. In both cases ascorbic acid eventually gets depleted (both mechanisms are depicted in Fig. 7).

As explained in Section 3.2, the establishment of the equilibrium between Fe^{2+} and Fe^{3+} via reactions with preformed LOOH gives a rapid drop in the oxygen concentration. It is not clear which mechanism is responsible for the large drops in the oxygraphic measurements, as both ascorbic acid oxidation by Fe^{2+} and lipid oxidation mediated by Fe^{2+} which is generated by ascorbic acid lead to rapid oxygen consumption. There is a likely possibility that both mechanisms run simultaneously. Measurement of primary and secondary oxidation products after the reaction could give an insight into which mechanism is prevailing; such measurements were however not performed in the frame of this study. Once ascorbic acid was depleted by interactions with the added iron, the rapid oxygen consumption stopped and the subsequent oxygen uptake was comparable to the control (demonstrated in Fig. 6A). This is in accordance with the above mentioned mechanisms.



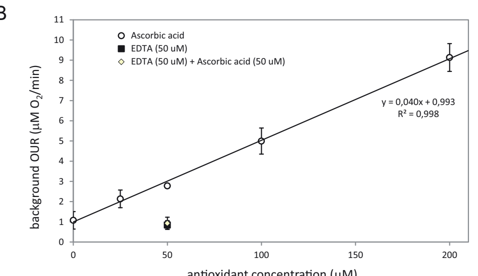


Figure 6. (A) Oxygen concentration curves with addition of ascorbic acid and Fe^{2+}, and only Fe^{2+} (control), (B) background OUR in emulsions with different concentrations of ascorbic acid (circles) and in emulsions containing EDTA (50 μM) (squares), and EDTA (50 μM) plus ascorbic acid (50 μM) (diamonds). The results are the mean values ± SD ($n = 3$–6).

In the study of Fukuzawa et al., peroxidation of liposomes made from egg yolk phosphatidylcholine (PC) induced by addition of ascorbate-Fe^{2+} complex was studied. Ascorbic acid was only slightly oxidized in liposomes from which pre-

Figure 7. Interactions of ascorbic acid with iron ions (adapted from [44, 45]).

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existing lipid hydroperoxides (LOOH) were removed by treatment with triphenylphosphine (TPP), but was oxidized extensively in liposomes not depleted of LOOH [46]. Lipid oxidation mediated by decomposition of LOOH by Fe^{2+} which was re-generated by ascorbic acid was attributed to this behavior. In the study of Yamamoto et al. the rate of oxidation of methyl linoleate micelles decreased as the oxidation proceeded and Fe^{2+} was oxidized to Fe^{2+} due to establishment of the equilibrium between Fe^{2+} and Fe^{3+}. Addition of ascorbic acid again accelerated the oxidation which was also explained by re-establishment of Fe^{2+} by ascorbic acid [47]. These findings are in favor of the pro-oxidant effect of ascorbic acid coupled to the reduction of Fe^{3+} rather than ascorbic acid oxidation by iron without involvement of lipids.

It should be mentioned that when ascorbic acid was added to the emulsion, an increase in the background OUR was observed (Fig. 6A), and the increase was proportional to the ascorbic acid concentration (Fig. 6B). One plausible explanation for this phenomenon could be a contamination of the emulsion by transition metals, which interacted with the added ascorbic acid, as described above. When EDTA (50 μM) was added prior to ascorbic acid, i.e., the endogenous metals were chelated, the increase in the background OUR after addition of ascorbic acid was no longer observed and the OUR remained the same as the one for only EDTA (Fig. 6B). The EDTA alone reduced the background OUR (by 30–70%) which also indicated involvement of endogenous metals in the peroxidation of the emulsion. The endogenous metals were not quantified, but their presence needs to be kept in mind when evaluating the effects of the added antioxidants. Ascorbic acid was not the only antioxidant which significantly affected the background OUR (R_B). The same phenomenon was observed for α-Tocopherol (discussed in Section 3.8).

3.7 β-Carotene and astaxanthin

Carotenoids are known to reduce photo-induced lipid oxidation by quenching reactive singlet oxygen, and to inhibit autoxidation of lipids by scavenging lipid free radicals [30, 48]. The effects of three different concentrations of β-carotene and astaxanthin (100, 200, and 300 μM (lipid based)) on the OUR and the equilibrium drop in the emulsions are shown in Fig. 8A (β-carotene) and Fig. 8B (astaxanthin). Astaxanthin slightly reduced the OUR, whereas β-Carotene slightly increased the OUR at all concentrations, but the effects were not significantly different from the control. Astaxanthin slightly reduced the equilibrium drop at all concentrations, while the drop was not significantly affected by β-carotene. In addition, no difference was found between the effect of astaxanthin on Fe^{2+}- and Fe^{3+}-mediated oxidation (Fig. 8B). Even though astaxanthin was found slightly better as an antioxidant in the emulsions than β-carotene, altogether, the two carotenoids had a marginal effect on iron-mediated oxidation in the emulsions.

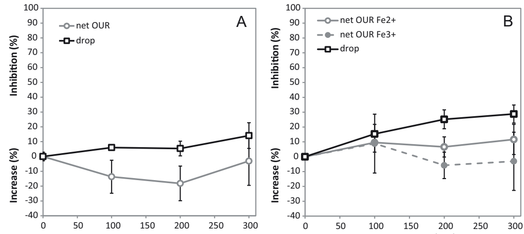

Figure 8. The effect of different concentrations of astaxanthin (graph A) and β-carotene (graph B) on OUR (circles) and the equilibrium drop (squares) in emulsions when Fe^{2+} (25 μM, empty circles) and Fe^{3+} (25 μM, full circles) (the latter only for astaxanthin) was used as a prooxidant. Positive values indicate inhibition (%), negative values indicate increase (%). The results are the mean values ± SD (n = 4–6).

The main difference between the two compounds is the presence of a keto-group and a hydroxyl group on the two β-ionone rings (Fig. 1) in the molecule of astaxanthin, which makes it more polar than β-carotene. This may have an influence on the location of the carotenoids in the emulsion. Shibata et al. found considerable differences between β-carotene and astaxanthin regarding their molecular packing and orientation in phospholipid layers. Astaxanthin showed a greater miscibility in phospholipid layers than β-carotene [49]. A tighter packing of astaxanthin in the phospholipid interface could give it an advantage in respect to radical scavenging abilities due to a better proximity to lipid radicals generated in the interphase. Astaxanthin also reduced the magnitude of the equilibrium drop. This could be attributed to scavenging of lipid radicals formed during the drop after addition of Fe^{2+}.

Carotenoids are efficient singlet oxygen scavengers. This mechanism is probably not of major importance in the emulsions since singlet oxygen is usually formed in the presence of photosensitizers, such as chlorophyll [4], which were unlikely to be present in the emulsions. To verify this, a series of experiments were performed in the dark. No difference was observed between the OUR measured in the ambient light and the dark (data not shown).

3.8 α-Tocopherol alone and with EDTA

The effect of different concentrations of α-tocopherol (100, 200, and 300 μM (lipid based)) on the net OUR and the equilibrium drop in Fe^{2+}(25 μM)-mediated oxidation are shown in Fig. 9A. Increasing concentrations of α-tocopherol in the emulsions increased the net OUR. There was a significant linear correlation between the concentration of α-tocopherol and the increase in the oxidation rates (shown in Fig. 9A). On the other hand, the drop was significantly reduced and the reduction increased with increasing concentrations of α-tocopherol, from 69% at 100 μm to 82% at 300 μM.

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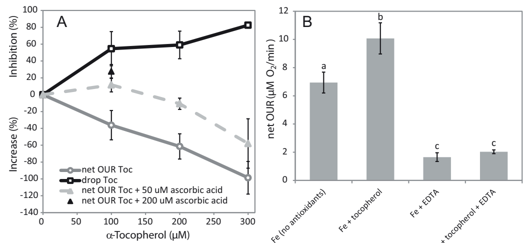

Figure 9. (A) The effect of different concentrations of α-tocopherol on the OUR (circles and triangles) and the equilibrium drop in oxygen concentration (squares) in lipid oxidation induced by Fe^{2+} (25 μM). Triangles represent emulsions containing ascorbic acid in addition (light triangle = 50 μM, dark triangles = 200 μM). Positive values indicate inhibition (%), and negative values increase (%). (B) The net OUR in the emulsions containing 100 μM α-tocopherol in the presence or absence of Fe^{2+} (25 μM), in the latter case the iron is chelated by EDTA (50 μM). The results are the mean values ± SD ($n = 3$–6). Values with different letters are significantly different ($p = 0.05$).

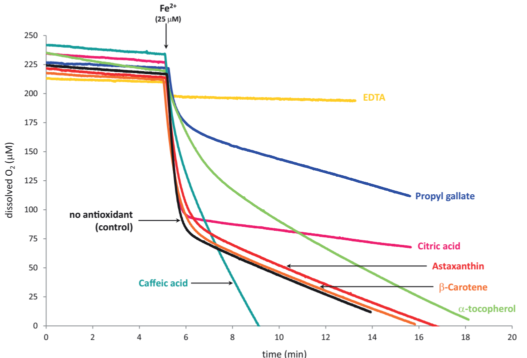

Figure 10. Direct comparison of representative oxygen consumption curves in10% w/v emulsions containing α-tocopherol, β-carotene, astaxanthin, propyl gallate, caffeic acid (each at 100 μM), EDTA and citric acid (each at 50 μM), and no antioxidant (control) in Fe^{2+} (25 μM)-mediated oxidation.

The antioxidant activity of tocopherols is related to their ability to donate hydrogen to the lipid peroxyl radicals (LOO$^{\bullet}$) leading to the formation of a lipid hydroperoxide and several resonance stabilized structures of tocopheroxyl radicals. Tocopheroxyl radicals are very reactive toward other radicals and in the presence of sufficient amounts of lipid alkoxyl, peroxyl, and alkyl radicals adducts with these radicals will be formed. The tocopheroxyl radicals can sometimes also take part in prooxidative reactions [50].

Tocopherols and tocopheroxyl radicals have the ability to reduce transition metals to the more catalytically active states [19]. Yamamoto et al. found that α-tocopherol enhanced oxidation in aqueous lipid dispersions when the oxidation was induced by Fe^{3+}. The authors observed the disappearance of α-tocopherol together with the formation of Fe^{2+} [51]. The red-ox cycling between Fe^{2+} and Fe^{3+} aided by LOOH results in continuous formation of Fe^{3+} which then can be reduced back to Fe^{2+} by α-tocopherol and the tocopheroxyl radicals. This agrees well with the OUR measurements. The prooxidative behavior of α-tocopherol observed in the emulsions was therefore hypothesized to be caused by reduction of Fe^{3+} to Fe^{2+} by α-tocopherol.

To verify this, EDTA was added to the emulsions containing α-tocopherol to deactivate both the endogenous metals and the added iron. The pro-oxidant activity of α-tocopherol was no longer observed when the metals were deactivated (Fig. 9B) which strongly suggest that iron reduction by tocopherol occurred in the emulsion. Another indication of interactions of α-Tocopherol with metals was an increased background OUR observed in the emulsions containing α-Tocopherol, compared to a control without antioxidants (shown in Fig. 10). The increased background OUR could be a result of α-Tocopherol reducing endogenous metals, which were expected to be present, as discussed in Section 3.6.

α-Tocopherol is considered to be an efficient lipid radical scavenger. Therefore chain-breaking reactions may have taken place alongside, even though the oxidation rates were increased due to red-ox cycling of iron. The reduction of the equilibrium drop favors this hypothesis. α-Tocopherol may scavenge the radicals formed during the initial reaction between Fe^{2+} and LOOH after the addition of Fe^{2+}. Elimination of these radicals would diminish the drop producing tocopheroxyl radicals, while the intact tocopherol could still be involved in the reduction of Fe^{3+} to Fe^{2+} after the drop.

3.9 α-Tocopherol with ascorbic acid

The antioxidant activity of tocopherols has been shown in several studies to increase when tocopherols and ascorbic acid were used together. This synergistic effect has been explained by the reducing abilities of ascorbic acid, which enable regeneration of tocopherols from the tocopheroxyl radicals. The interaction between the two compounds is believed to take place at the surface of the emulsion droplets since ascorbic acid resides in the aqueous phase while tocopherol is located within the lipid phase [52, 53]. The influence of pro-oxidants on this co-operation is often neglected.

The effect of ascorbic acid (50 μM) on the OUR in the emulsions with different concentrations of α-tocopherol as well as the effect of α-tocopherol alone in Fe^{2+} (25 μM)-mediated oxidation is shown in Fig. 9A. Addition of Fe^{2+} to the emulsions with ascorbic acid and α-tocopherol resulted in large equilibrium drops (curves not shown), similar to those

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observed in emulsions with only ascorbic acid (described in Section 3.6). The net OUR which followed after the drops were however reduced in respect to the OUR measured for tocopherol alone. A marginal inhibition ($12 \pm 8\%$) of the net OUR occurred at $50\,\mu M$ ascorbic acid and $100\,\mu M$ α-tocopherol. When the concentration of ascorbic acid was increased to $200\,\mu M$ the inhibition was improved to $28 \pm 6\%$ (Fig. 9A). Interactions between ascorbic acid and iron, tocopherol and iron, and tocopherol and ascorbic acid simultaneously seem to determine the final OUR. The experiments suggest that ascorbic acid may be advantageous for regeneration of tocopherol radicals when it is present in excess to both iron and α-tocopherol.

In Section 3.8, it is hypothesized that the prooxidative and antioxidative mechanisms of α-tocopherol take place simultaneously, since the equilibrium drop in the oxygen concentration was reduced while the net oxidation rates were increased in the emulsions containing α-tocopherol. The lower net oxidation rates found when ascorbic acid was added to the emulsions with α-tocopherol agrees well with this hypothesis, as ascorbic acid is known to prolong the free radical scavenging activity of α-tocopherol via regeneration of α-tocopherol [50, 52]. This effect seems however valid only at some favorable ratios between ascorbic acid, iron, and tocopherol, as shown in Fig. 9A. The dual character of ascorbic acid in the presence of metals needs to be kept in mind when evaluating the effects of ascorbic acid and its activity coupled to the regeneration of tocopherols.

The study shows that interactions between LMW iron and antioxidants have a significant impact on lipid oxidation in marine emulsions stabilized with phospholipids. The location of the antioxidants in multiphase systems is of importance when considering their antioxidative activities. According to the much disputed polar paradox [54], the most efficient antioxidants in oil-in-water emulsions should be the non-polar antioxidants, i.e., tocopherols and carotenoids, and the least activity should be observed for propyl gallate and caffeic acid. Propyl gallate was shown to be the best antioxidant for emulsions containing LMW iron in this study while caffeic acid showed prooxidative behavior. The outcomes therefore show clearly that the postulations in the polar paradox are too simple and among other aspects do not take into account the interactions between the antioxidants and prooxidants, such as iron.

This study also shows, that the interactions between LMW iron (and possibly other metals or types of pro-oxidants, such as heme-proteins) and various dietary antioxidants (possibly also natural extracts, novel antioxidants, such as phenolipids) and their impact on lipid oxidation in emulsified systems may be screened by the means of the oxygen uptake method. This approach could be useful for designing effective antioxidant strategies for specific lipid-rich systems and for systematic investigation of anti- and pro-oxidant mechanisms, as demonstrated in a study by Roginsky et al. [55].

Caffeic acid, ascorbic acid, and α-tocopherol exhibited prooxidative behavior in the emulsions at the tested concentrations and ratios to Fe^{2+}. The prooxidative effects were attributed to their ability to reduce Fe^{3+} to Fe^{2+}. The prooxidative effect of caffeic acid was greater than that of α-tocopherol which could be due to a greater reducing power of caffeic acid. Both caffeic acid and α-tocopherol had an inhibitory effect on the equilibrium drop in the dissolved oxygen concentration, which could be a result of the combination of scavenging of free radicals generated during the drop and of iron chelation since both caffeic acid and propyl gallate have been reported to have metal chelating properties. These have been attributed to their catechol and galloyl groups, respectively. To illustrate the effects of antioxidants in LMW iron-mediated oxidation, a direct comparison of the oxygen concentration curves in emulsions containing the antioxidants is shown in Fig. 10. Even though the antioxidant mechanisms might be present to some degree, they are eventually suppressed by the reducing abilities of the compounds which favor iron reduction, i.e., the prooxidant effects. The results also underline that which mechanism prevails is dependent on the concentration of both the antioxidants and pro-oxidants.

By recording the dissolved oxygen concentration in the emulsions and quantification of the rates of dissolved oxygen consumption, it was verified that the interactions between iron and antioxidants had a significant impact on the lipid oxidation in the 10% oil-in-water marine emulsions stabilised with phospholipids. The interactions of iron ions, which may be added or present endogenously, with antioxidants must therefore be considered when interpreting the effects of different antioxidants on lipid oxidation. This study shows that it is possible to screen these interactions by quantification of the dissolved oxygen consumption.

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PAPER IV

Influence of human gastric juice on oxidation of marine lipids – *in vitro* study

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Influence of human gastric juice on oxidation of marine lipids – *in vitro* study

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ABSTRACT

This study evaluates whether marine lipids can oxidise in acidic stomach environment and whether authentic gastric juice has the potential to act as a pro- or anti-oxidative medium. Oxidation of herring lipids in emulsions and liposomes was followed in *in vitro* digestion models containing authentic human gastric juice, and compared to models containing hydrochloric acid solution. Peroxide value, concentration of thiobarbituric acid reactive substances and oxygen uptake rate increased in all the models during 2.5 h incubation at pH 4 and 37 °C in darkness. The markers showed no difference between oxidation in gastric juice and hydrochloric acid solution. Gastric juice reduced the prooxidant activity of iron ions measured as oxygen uptake rate, but did not reduce the activity of methemoglobin. Berry juice, green tea, red wine, and caffeic acid reduced oxygen uptake in the acidic environments while coffee, ascorbic acid and orange juice increased oxidation. Beverages accompanying foods containing marine lipids will therefore affect the course of post-prandial lipid oxidation.

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1. Introduction

The importance of marine long-chain omega-3 polyunsaturated fatty acids (LCPUFA) in the diet, especially eicosapentaenoic (EPA, C20:5 n3) and docosahexaenoic acid (DHA, C22:6 n3), has been well established during the past two decades. Positive physiological effects in areas of body development and function, immunity and health maintenance, as well as therapeutic benefits, such as preventing heart, coronary, mental and chronic diseases, have been reported in a number of studies and extensively reviewed (Eduardo, 2010; Gorjão et al., 2009; Narayan, Miyashita, & Hosakawa, 2006).

On the other hand, lipid radicals and oxidised derivatives of omega-3 fatty acids (e.g. hydroperoxides, hydroperoxy epidioxides, core aldehydes, and epoxy compounds) and other products of oxidative degradation (e.g. low molecular weight aldehydes and hydroxyl alkanals) are believed to be cytotoxic and linked to development of cancer, atherosclerosis, thrombosis, inflammation and neurodegenerative and other diseases (Breivik, 2007; Gerhard, 2006; Guillen & Goicoechea, 2008; Kanner, 2007; Spickett & Dever, 2005; Turner, McLean, & Silvers, 2006). These compounds can be transferred from oxidised lipids into the bloodstream (Staprans, Rapp, Pan, Kim, & Feingold, 1994). The oxidative state of omega-3 fatty acids entering the blood stream therefore appears crucial for the overall impact of marine lipids on health (Turner et al., 2006).

The risk of consuming deteriorated marine lipids is justified considering the high susceptibility of LCPUFA to oxidation (Breivik, 2007). Oxidation occurring in the food matrix itself is however not the only health threat. Several studies have proposed that the gastrointestinal (GI) tract could be an excellent environment for enhancing oxidation of lipids and other food constituents before they are metabolised, in other words undergoing post-prandial oxidation (Halliwell, Zhao, & Whiteman, 2000; Kanner, 2007; Kanner & Lapidot, 2001). During post-prandial oxidation, the cells of the GI tract are likely to be exposed to the cytotoxic molecules, reactive lipid radicals and reactive oxygen species accompanying oxidation (Halliwell et al., 2000), which may aid development of cancers in the GI tract. Preventing lipid oxidation during the time the lipids are retained in the stomach may therefore reduce the overall amount of oxidised lipids entering the bloodstream and prevent processes damaging the cells of the GI tract.

Assessing the degree of lipid oxidation is not straightforward when it comes to the GI tract, as extensively reviewed by Hur, Lim, Decker, & McClements, 2011. In most published studies,

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simulated GI conditions have been employed, with respect to the composition and concentrations of acids, enzymes and salts. Human gastric juice contains a wider spectrum of components than simulated formulations, including mucous and saliva. The use of authentic human gastric juice has therefore been recommended to establish more accurate conditions for *in vitro* digestion studies (Ulleberg et al., 2011), since the occurrence and extent of post-prandial oxidation might be at least partially governed by the authenticity of the gastric juice.

To the best of our knowledge, the influence of authentic human gastric juice on oxidation of vulnerable marine lipids has not been investigated. Lipid hydroperoxide formation in heated muscle tissue and linoleic acid emulsions in simulated gastric juice was found to be higher at pH 3.0 than at pH 5.0 in a study of Lapidot, Granit, & Kanner, 2005a. Protection of lipids from oxidation by various dietary antioxidants in *in vitro* GI models had largely positive results in a number of studies, suggesting consumption of antioxidant rich food alongside food rich in lipids (Gorelik et al., 2005; Gorelik, Ligumsky, Kohen, & Kanner, 2008b; Kerem, Chetrit, Shoseyov, & Regev-Shoshani, 2006; Tagliazucchi, Verzelloni, & Conte, 2010).

Directly after the food intake and during prolonged eating, the gastric juice is diluted by mucous, saliva and liquids from food. The pH is increased from its basal value (1–2) and can reach value up to 6 before resuming its initial value under complete stomach emptying and fasting (Kalantzi et al., 2006). This stage may last for up to 2.5 h, depending on the meal composition, proportion of liquids in the food, and the time span of eating (Hur et al., 2011).

Lipid oxidation is facilitated by oxygen which needs to be available in a closed stomach system. It has been proposed that saliva, masticated food, liquids and swallowed air bring enough oxygen into the stomach, which can then facilitate post-prandial oxidation (Kanner & Lapidot, 2001).

In the present study oxidation of herring lipids in liposomes and emulsions was followed in *in vitro* digestion models at pH 4.0 containing authentic human gastric juice, and compared to models lacking gastric juice components apart from hydrochloric acid. The aim was to evaluate whether marine lipids are prone to oxidation in the acidic gastric juice environment and whether gastric juice itself has the potential to act as a pro- or anti-oxidative medium. The effect of dietary pro- and antioxidants, involving several beverages rich in antioxidants, on oxidation under the stomach conditions was also investigated.

2. Materials and methods

2.1. Materials

Authentic human gastric juice (GJ) was kindly donated by GastroLab in St. Olavs Hospital (Trondheim, Norway), after it was collected from a healthy adult undergoing a pentagastrin test in January 2011 (ca. 200 ml, pH ~1). The fresh gastric juice was filtered (589/1 filter paper, Whatman GmbH, Dassel, Germany) to remove thick mucous and remnants of dispersed food, mixed and divided into ca. 20 ml portions which were stored at −80 °C until needed. Before experiments, a necessary amount was allowed to thaw at ambient temperature.

Mature roe from Norwegian spring spawning herring (*Clupea harengus*) obtained from Grøntvedt Pelagic (Uthaug, Norway), was used for extraction of marine phospholipids. Before extraction (described in Section 2.6), the roe glands were kept frozen at −40 °C. Herring (*Clupea harengus*) oil was produced by SINTEF Mobile processing plant in November 2010 from ultra fresh herring rest raw material obtained from Grøntvedt Pelagic (Uthaug, Norway). The crude herring oil was kept frozen at –30 °C until further processing (described in Section 2.3).

Green tea (*Camellia sinensis*) in bags (Ecologic Green Ceylon Tea, Confecta AS, Oslo, Norway), ground black coffee (Friele Frokost Kaffe, Oslo, Norway), red wine (Marqués de Schivé, Tempranillo Crianza, 2007, produced by Vicente Gandía Plá SA, Valencia, Spain), 100% orange juice from concentrate (Nora Familiens beste appelsinjuice, Stabburet AS, Kolbotn, Norway) and a composite berry juice from concentrate (Nora Familiens beste skogsbærjuice med drue og eple, containing 50% grapes, 25% apple, 8% blueberry, 7% blackberry, 5% raspberry and 5% pomegranate, and 300 mg/l ascorbic acid, Stabburet AS, Kolbotn, Norway) were purchased at a local market.

2.2. Chemicals and reagents

Caffeic acid, ascorbic acid, 2-(*N*-morpholino)ethanesulfonic acid (MES), bovine methemoglobin (metHb), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), trichloracetic acid (TCA), sodium dithionite ($Na_2S_2O_4$), sodium chloride, 1,1,3,3-tetraethoxypropane, 0.1 mol sodium thiosulphate ($Na_2S_2O_3$) aqueous solution, potassium iodide, potassium iodate, boron trifluoride (BF_3), iron standard (Titrisol), ethylenediaminetetraacetic acid (EDTA), and lipid classes standards were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Hydrochloric acid (HCl), potassium chloride, ammonium thiocyanate (NH_4SCN), ferrous sulphate ($FeSO_4 \cdot 7H_2O$) and all solvents were supplied by Merck KGaA (Darmstadt, Germany). Anhydrous ferric chloride ($FeCl_3$) was purchased at Riedel de Haën (Seelze, Germany). Sodium hydroxide (NaOH), ferrous chloride ($FeCl_2 \cdot 4H_2O$) and all HYDRANAL products (Karl-Fisher reagent – Composite 2, dry methanol, dry chloroform, and water-in-methanol standard: 5 mg H_2O/ml) were obtained from Fluka Chemie (Buchs, Germany). Nitrogen (99.99% N_2) and helium gas (99.99% He) were provided by AGA AS, Oslo. Standards of fatty acid methyl esters and lipid classes were purchased at Nu-Check Prep Inc. (Elysian, MN, USA). Phospholipid standards were purchased at Avanti Polar Lipids Inc. (Alabama, USA). All chemicals and solvents were of analytical grade, except for solvents used in TLC–FID, GC–FID and HPLC–CAD analyses, which were of chromatography grade. Distilled water was used for preparing aqueous solutions.

2.3. Polishing of crude herring oil

Crude herring oil was allowed to thaw in a warm water bath (ca. 40 °C) before washing with distilled water. Briefly, 10% (w/w) of boiling water was added to the crude oil (in relation to the oil mass), and thoroughly mixed for 10 min in a water bath held at 70 °C (Crexi, Monte, Soares, & Pinto, 2010). The warm mixture was then centrifuged (7000 *g*, 10 min, 40 °C) and the oil phase was collected, divided into 50 ml portions and stored at −20 °C until needed. The impact of the polishing step on the oxidation status and moisture content in the oil is discussed in Section 3.1.

2.4. Peroxide value in herring oil

Peroxide value (PV) in raw and polished herring oil was determined by the iodometric titration method according to a titration application issued by Radiometer Analytical (TTIP02-01AFD/2002-06A, 2002). The application is based on the AOCS official method for PV determination in edible oils (Cd 8b-90). The titration end point was assessed potentiometrically, using an automatic titrator (TitraLab980) coupled with a single platinum electrode (M21Pt) and a reference electrode (REF 921) (all equipment produced by Radiometer Analytical ASA, Copenhagen, Denmark). The minimum and maximum speed of a standardised titrant (0.01 M

$Na_2S_2O_3$) (TTEP01-08MIN/2001-05A, 2002) addition was 0.2 ml/min and 3.0 ml/min, respectively; the smoothing parameter was set to 1. The analysis was performed with five parallels, and the results are expressed in mmol LOOH/kg as a mean value + standard error (SE). The calculation is shown in Eq. (1)

$$\mathrm{PV}\left(\frac{\mathrm{mmol}}{\mathrm{kg}}\right) = \frac{c \times (V_s - V_b) \times 2}{G} \times 1000 \quad (1)$$

C is the concentration of titrant (average value, $n = 3$) (mol/L), V_s is the titrant consumption for sample (ml), V_b is the titrant consumption for blank (ml), G is the amount of lipids for analysis (g), 1000 is a conversion factor for units, and 2 is the stoichiometric molar ratio between LOOH and $Na_2S_2O_3$.

2.5. Moisture content in herring oil

Moisture level in the crude and polished herring oil was determined by Karl-Fisher titration according to a titration application issued by Radiometer Analytical (T550VKF041, 2002) using HYDRANAL chemicals. The titration end point was assessed potentiometrically by an automatic titrator (TitraLab980) coupled with a double platinum electrode (M231Pt2) (all equipment produced by Radiometer Analytical ASA, Copenhagen, Denmark). Water-in-methanol standard (5 mg H_2O/ml) was used for standardisation of the commercial Karl-Fisher reagent. The accuracy of the method was verified by measuring fish oil with a known moisture level. The analysis was performed with five parallels and the results are expressed in % (w/w) of water in the oil ± SE.

2.6. Isolation of phospholipids from roe

The herring roe was allowed to thaw overnight at 4 °C. The extraction of total lipids from the roe was performed according to the method of Bligh & Dyer, 1959. The phospholipids (PL) were isolated from the total lipids by precipitation in cold acetone, as described by Kates, 2010 and modified by Mozuraityte, Rustad, & Storro, 2006. The precipitation was performed two times in order to increase purity. The final precipitate was stored at −20 °C as PL–chloroform solution and used for experiments.

2.7. Purity of oil and isolated phospholipids

The lipid classes in the lipids were determined by a thin layer chromatography with flame ionisation detector system (Iatroscan TLC-FID analyzer MK-6, Mitsubishi Kagaku Iatron Inc., Tokyo, Japan). Briefly, lipids dissolved in chloroform (10 mg/ml) were injected (3 μl) on silica coated quartz rods (Chromarod-SIII, Mitsubishi Chemical Medience, Tokyo, Japan). The rods were placed into a tank with vapours of saturated NaCl solution for 8 min. Afterwards the tips of the rods were dipped into *n*-hexane/diethyl ether/formic acid (85:15:0.04, v/v/v) for 27 min inside a development tank. The solvent was evaporated from the rods and the rods were scanned. Lipid classes were characterised by comparison to the retention times of commercial standards run at the same conditions. The accuracy of the method was verified by measuring commercial standards of lipid classes. Each sample was analysed in duplicate and % of total peak area was calculated for each class. The results are average values with maximum coefficient of variation 13%.

2.8. Fatty acid profile

Ten mg of lipids were dissolved in chloroform containing a known amount of internal standard (21:0). The chloroform was completely evaporated by N_2 gas and the oil was redissolved in 1 ml of 0.5 M methanolic NaOH, hydrolysed for 15 min at 100 °C, and cooled. Two millilitres of 10% BF_3 in methanol was added and the mixture was incubated for 5 min at 100 °C, and cooled. Afterwards, 1 ml of hexane was added and the mixture was incubated for 1 min at 100 °C, and cooled. Finally, 0.5 ml of hexane and 2 ml of saturated NaCl solution was added, the mixture was vortexed and centrifuged at 2000 rpm for 3 min (Universal 16A centrifuge, Hettich Zentrifugen, Tuttlingen, Germany). The organic phase containing fatty acid methyl esters (FAME) was collected and washed two times with 0.5 ml of hexane.

The fatty acid composition in the methylated samples was analysed by an Agilent Technologies 7890A gas chromatograph with flame ionisation detection (GC–FID) system equipped with 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA). The detector temperature was held at 270 °C, and the flame was maintained with 25 ml/min hydrogen gas and 400 ml/min air. Chromatography was carried out using a Cp-wax 52CB, 25 m × 0.25 mm with id = 0.2 μm column (part no. CP7713, Agilent Technologies). Helium was used as the carrier gas at a flow rate 1.5 ml/min. GC inlets were held at 250 °C. The initial oven temperature was held at 80 °C and gradually increased by 25 °C/min until it reached 180 °C, followed by a 2 min hold. Then the temperature was increased by 2.5 °C/min to 205 °C, followed by a 6 min hold, after which the temperature was increased by 2.5 °C/min to 215 °C, followed by a final hold of 4 min. The total analysis time was 31 min. Fatty acids were characterised by comparison to the retention times of commercial standards and quantified by internal standard. The accuracy of the method was verified by comparison of FA profiles of selected marine oils against profiles assessed by accredited laboratories. Each sample was analysed in duplicate and % of total peak area was calculated for each fatty acid. The results are average values with maximum coefficient of variation 3%.

2.9. Analysis of phospholipid classes by reverse phase HPLC–CAD

The composition of the isolated PL was analysed by the Agilent 1260 Infinity HPLC system (Agilent Technologies, Germany) coupled to the ESA Corona Charged Aerosol Detector (CAD) (Thermo Scientific/Dionex, USA). The PL were dissolved in isopropanol (1 mg/ml) and separated on Agilent Prep-SIL Scalar 10 μm column, 4.6 × 150 mm (Agilent Technologies, Santa Clara, CA, USA) kept at a constant temperature (22.0 ± 0.8 °C). For the isocratic elution a ternary gradient having a constant flow rate of 1.25 ml/min and consisting of degassed solvents A = *n*-hexane, B = 2-propanol, and C = deionised water (MiliQ) was used with the following timetable: at 0.00 min 40:59:1 (%A:%B:%C); at 3 min 40:54:6; at 18.00 min 40:50:10; at 18.01 min 40:59:1; and at 23 min 40:59:1. The sample temperature was 4 °C and the injected volume was 10 μl. The phospholipid classes were identified by comparison to the retention times of commercial standards and quantified from standard curves measured at the same conditions. Each sample was analysed in duplicate and % amount (w/w) was calculated for each PL class. The results are average values with maximum coefficient of variation 3%.

2.10. Preparation of liposomes

Liposomes were prepared fresh before each set of experiments as described in our earlier papers (Kristinova, Mozuraityte, Storro, & Rustad, 2009; Mozuraityte et al., 2006). Briefly, an aliquot of chloroform solution of marine PL was evaporated to dryness with a stream of N_2 gas; the residual solvent was completely evaporated under vacuum (2 h). The dried mass of PL was then dissolved in a 5 mM MES solution (pH 7.5), to a concentration of 60 mg/ml (6.0% lipids, w/v), and the solution was sonicated in a 25 mm (diameter) glass tube using a 12 mm (diameter) sonication probe (Vibra Cell, Sonics & Materials Inc., Newtown, CT, USA) under the following

conditions: pulse: 6 s, amplitude: 50%, total sonication time: 4.0 min. The liposome dispersion was kept on ice both during and after the sonication.

2.11. Preparation of oil-in-water emulsion

Herring roe PL were dissolved in the polished herring oil to a concentration 91 mg/g, by blending the oil with the PL–chloroform solution. Chloroform was evaporated from the mixture by a rotavapor (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) (1 h, 30 °C, 30 mbar) and the mixture was kept frozen at −20 °C until needed. 22% oil-in-water emulsion (w/v) was prepared fresh before each set of experiment by homogenising (Ultra-Turrax T25 with 10 mm (diameter) dispersing rod, IKA-Werke GmbH & Co. KG, Stafen, Germany) 5.5 g of the PL–oil mixture with 19.5 ml of 5 mM MES solution, pH 7.5. The homogenisation lasted 4 min with gradual increase of blade rotation, from 8,000 to 20,500 rpm. The emulsion was kept in dark at 4 °C until needed.

2.12. Acidity of gastric juice

Gastric juice acidity was determined by potentiometric titration against standardised 0.1 M NaOH, using an automatic titrator (TitraLab980, Radiometer Analytical ASA, Copenhagen, Denmark), and a combination glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, USA), which was calibrated against standard buffer solutions with pH 4.0 and 7.0. A titration application was followed (TTEP01-01PHR/2001-10A, 2002). The analysis was performed with five parallels and the results were expressed in M HCl as a mean value ± SE.

2.13. pH verification

The pH of solutions, liposomes and emulsions, drinks and experimental mixtures was measured by a TIM900 Titrator manager (TitraLab, Radiometer Analytical AS, Copenhagen, Denmark) coupled with a combination glass electrode (LIQ-GLASS 238000/08, Hamilton Co., Reno, USA), which was calibrated daily against standard buffer solutions at pH 4.0 and 7.0 at 22 °C.

2.14. Preparation of gastric juice (GJ)–lipid models

GJ–liposome model: an aliquot of 6% liposomes was mixed with an aliquot of gastric juice and aliquots of NaOH solutions (0.01–1.5 M), making up a final mixture having pH 4.00 ± 0.05 and consisting of 2.5% PL as liposomes (w/v) and 50% gastric juice (v/v).

GJ–emulsion model: an aliquot of 22% oil-in-water emulsion was mixed with an aliquot of gastric juice and aliquots of NaOH solutions (0.01–1.5 M) making up a final mixture having pH 4.00 ± 0.05 and consisting of 10% emulsified lipids (w/v) and 50% gastric juice (v/v). The mixtures were kept on ice and gently stirred during and after preparation. Twelve millilitres and 35 ml final volumes were prepared.

Each GJ–lipid model had two control models: Control 1 in which the gastric juice was substituted with 0.11 M HCl solution, and Control 2 in which the lipids were substituted with 5 mM MES buffer, pH 7.5. The concentration of HCl in Control 1 matched the acid concentration in the gastric juice. The Controls 2 were introduced in order to verify the contribution of GJ components to the colour formation in the PV and TBARS assays (Sections 2.16 and 2.17).

2.15. Oxidation experiments

For PV and TBARS measurements and to simulate stomach environment, 12 ml of each model was transferred into a plastic syringe equipped with a few glass beads to facilitate even mixing and tightly closed with a plunger and a cannula. Five millilitres and 10 ml of ambient air headspace were left in the syringes for liposomes and emulsions, respectively. The headspace volumes were chosen to establish conditions where O_2 availability from air was not a limiting factor for peroxidation during the whole incubation period. The syringes were mounted onto a carousel rotating at 10 rpm (Stuart rotator SB3, Barloworld Scientific Ltd., Stone UK) and placed into a dark laboratory incubator pre-heated at 37.0 ± 0.1 °C. One ml aliquots were taken from the syringes each 30 min for a period of 2.5 h and immediately analysed for either PV or TBARS. New batches were prepared for each method. A schematic figure of the experimental setup is shown in Fig. 1A.

2.16. Peroxide value in isolated phospholipids and emulsified lipids

Peroxide value (PV) in the isolated phospholipids and emulsified lipids was analysed by the ferric thiocyanate assay described by the International Dairy Federation (Standard_74A, 1991), and modified by Ueda, Hayashi, & Namiki, 1986 and Undeland, Stading, & Lingnert, 1998, with further modifications according to Mihaljević, Katušin-Ražem, & Ražem, 1996. The method was chosen because it requires small amounts of samples (mg).

Briefly, a 100 µl aliquot of liposomes/emulsion or lipids dissolved in 5-methylpentane was added to a mixture consisting of 5 ml 96% ethanol and 200 µl 4% BHT dissolved in ethanol. Afterwards, 200 µl of a reagent solution prepared by mixing equal volumes of 0.4 M ethanolic NH_4SCN and 4.5 mM $FeSO_4{\cdot}7H_2O$ in 2 M HCl was added. All solutions were deaerated by nitrogen gas. The absorbance was read at 500 nm against ethanol exactly 10 min after addition of the reagent solution. During the analysis, samples, the spectrophotometric mixtures and the reagent solution were kept on ice. A standard curve prepared with $FeCl_2{\cdot}4H_2O$ was used for quantification of results. Nine parallels were measured for isolated phospholipids. For emulsions/liposomes, five consecutive parallels were taken from the 1 ml aliquot sampled at each time point. No contribution of GJ to the colour formation was found (data not shown). The results were expressed in mmol LOOH/kg fat as a mean value SE. Eq. (2) shows the PV calculation:

$$\mathrm{PV}\left(\frac{\mathrm{mmol}}{\mathrm{kg}}\right) = \frac{(Abs - Abs_{bl}) \times V}{S \times 55.845 \times 100 \times G} \times 1000 \times 2 \qquad (2)$$

Abs is the absorbance of the sample, Abs_{bl} is the absorbance of the blank (average value, $n = 3$), V is the total volume of liposomes/emulsion (ml) or volume of 5-methylpentane in which the PL were dissolved, S is the slope of the standard curve (µg), G is the amount of PL used for preparing liposomes or the amount of PL dissolved in 5-methylpentane (g), 55.845 is the molar weight of iron (g/mol), 100 is the aliquot of liposomes or 5-methylpentane-PL solution used for analysis (µl), 1000 is the conversion factor for units, and 2 is the correction factor (Mihaljević et al., 1996).

2.17. Analysis of thiobarbituric acid reactive substances (TBARS)

TBARS values in the isolated phospholipids and herring oil were determined by the spectrophotometric method described by Ke and Woyewoda (1979). All amounts were reduced to one half relative to the given procedure. The analysis was performed with five parallels for oils and nine parallels for PL. The results are expressed in mmol TBARS/kg lipids as a mean value ± SE.

TBARS values in liposomes/emulsions were determined according to the method of McDonald & Hultin, 1987. The analysis was performed with five consecutive parallels taken from the 1 ml aliquot sampled at each time point. The TBARS measurements were increased by 6% (relative to MES solution) by gastric juice (data

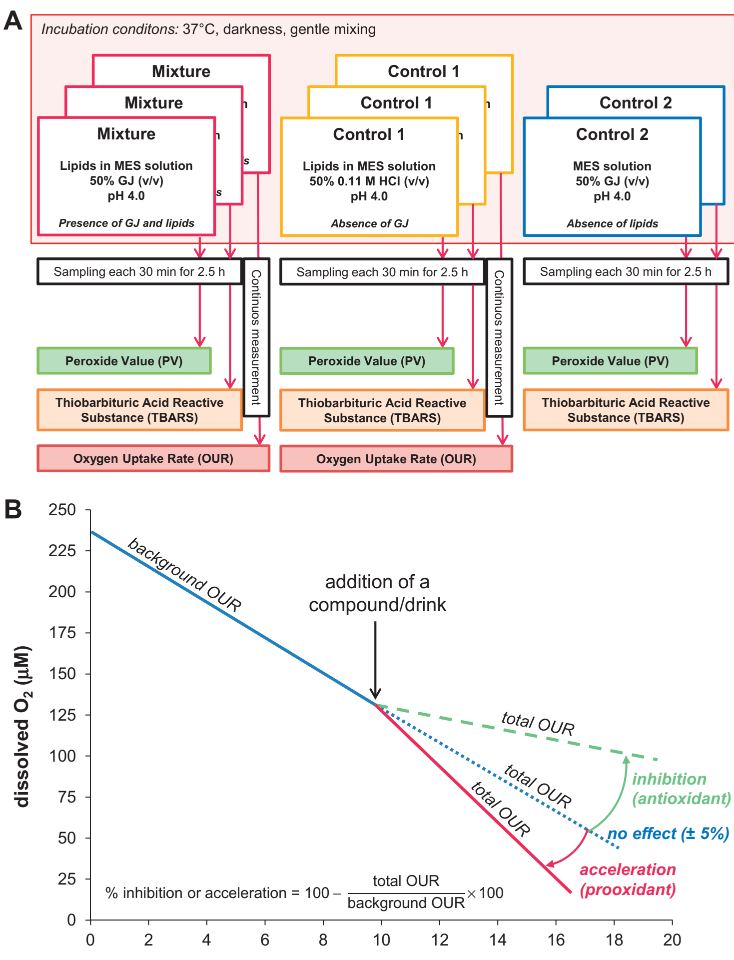

Fig. 1. (A) Experimental setup for determination of the peroxide value (PV), thiobarbituric acid reactive substances (TBARS) and oxygen uptake rate (OUR) in the gastric juice–lipid blends (Mixture) and the corresponding controls (Control 1 and Control 2); (B) schematic illustration and description of an oxygen uptake measurement in liposomes/emulsion with addition of an exogenous compound.

not shown). Therefore the TBARS values in the GJ–lipid mixtures were corrected for the colour development caused by the gastric juice itself. The results were expressed in mmol TBARS/kg fat as a mean value ± SE. Eq. (3) shows the calculation:

$$\text{TBARS}\left(\frac{\text{mmol}}{\text{kg}}\right) = \frac{Abs \times f}{\varepsilon \times L} \times \frac{V}{G} \qquad (3)$$

Abs is the absorbance of the spectrophotometric mixture, *f* is the dilution factor of the sample in the spectrophotometric mixture, ε is the absorption coefficient (156,000 l/mol cm), *L* is the length of the optical path (1 cm), *V* is the total volume of liposomes/emulsion (ml) and *G* is the mass of lipids in the total volume (kg).

2.18. Preparation of added compounds

Stock solutions of Fe^{2+} ($FeSO_4{\cdot}7H_2O$) and Fe^{3+} ($FeCl_3$) in 0.5 M HCl were prepared monthly and kept in dark; the pH of the solution was kept at 0.5 in order to maintain iron solubility and prevent iron precipitation. Work solutions were prepared fresh before experiments by diluting an aliquot of the stock solutions with 5 mM MES solution, pH 5.5. A work solution of bovine methemoglobin (metHb) was prepared fresh before experiments by dissolving metHb in 5 mM MES solution, pH 5.5. Stock solutions of antioxidants (caffeic acid, ascorbic acid) were prepared in 96% ethanol and stored at 4 °C. Work solutions were prepared daily by diluting an aliquot of the stock solutions with distilled water. Ethanol in these work solutions made up 2%. The work solutions (50 µl) were injected into the oxygraphic cells (described in Section 2.20). The final concentrations of the added compounds in the reaction mixtures were: 100 µM caffeic and ascorbic acid, 10 µM iron ions, and 10 µg/ml (0.16 µM) metHb.

2.19. Preparation and treatment of beverages

Black coffee was prepared by pressing 1.5 l of boiling water through 90 g of ground coffee beans using a kitchen coffee machine (Matic Twin, Bravilor Bonamat). Green tea was prepared by pouring one tea bag (2.2 g green tea) with 200 ml of 90 °C distilled

water and extracting the bag for exactly 3 min. Coffee and tea were prepared fresh before each set of measurements from the same batch of material. Red wine, berry juice and orange juice were mildly shaken before opening the bottle/cartons. New cartons/bottles were opened for experiments with liposomes, and new cartons/bottles from the same batch were opened for experiments with emulsions to assure freshness of the beverages. After opening, the bottles/cartons were stored in a fridge. 50 μl of the beverages were injected into the experimental mixtures. Dose-to-test volume ratio was 1:20 (v/v) for all the measurements, which represents a small glass (75 ml) diluted in 1.5 l stomach content; for red wine, the ratio was increased to 1:10 (v/v) to simulate one glass of wine (150 ml).

2.20. Oxygen uptake rate measurements

One millilitre of the liposomes/emulsion was transferred into a water-jacketed oxygraphic cell, in which the concentration of the dissolved oxygen was measured by the Clark polarographic oxygen electrode (Oxygraph system, Hansatech Instruments Ltd., Norfolk, UK) calibrated against O_2 saturated and O_2 depleted distilled water ($Na_2S_2O_4$ was used for depletion). The cell was equipped with a magnetic stirrer, wrapped by aluminium foil to block ambient light and closed with a plunger with a capillary opening preventing access of air oxygen and allowing injection of solutions. The concentration of the dissolved O_2 (μM) was continuously recorded as a function of time (min), giving oxygen concentration curves. As a measure of oxidation, oxygen uptake rates (OUR, μM O_2/min) were calculated from the curves using Oxyg32 software. In relation to OUR measurements, the following terminology is used throughout the text: background OUR = rate measured in the pure model, i.e. prior to injection of any exogenous liquid, total OUR = rate measured after the injection, net OUR = total OUR subtracted from background OUR.

In the experiments without addition of any external compound/beverage, the background OUR was measured at 10–20 min intervals for a period of 160 min. At OUR > 2 μM O_2/min, the dissolved oxygen in the experimental volume (1 ml) was completely consumed before the end of the incubation period. To be able to follow the oxygen uptake for the whole incubation period, the cell was opened when the concentration of the dissolved O_2 reached almost zero, and air was quickly infused into the liposomes/emulsions until O_2 concentration reached the saturation level. Afterwards, the cell was closed again without interruption of the oxygen uptake recording; this was performed before each complete O_2 depletion for as long as necessary.

The effect of added compounds/beverages was evaluated as % inhibition or increase of the background OUR. The duration of one experiment was ca. 30 min, and the compounds/beverages were added by after 10–15 min of background oxygen uptake recording. Freshly made liposomes were used for all experiments with added compounds. Due to a low background OUR in the emulsions, the emulsions were allowed to stand at room temperature for 24 h in order to increase the background OUR and better assess the effect of added compounds/beverages. Three cells were run simultaneously for each experiment and the result is given as a mean value ± SD. A schematic figure of an oxygen uptake recording is shown in Fig. 1B.

2.21. Statistical analysis

For assessment of significant difference between two values a two sided Student's *t*-distribution was used, employing MiniTab software. The level of significance was set at $p < 0.05$ (95%).

3. Results and discussion

3.1. Characterisation of lipids and human gastric juice

The lipids in this study originated from Norwegian spring spawning herring (*Clupea harengus*). Phospholipids (PL) were isolated from mature herring roe and crude oil was pressed from an

Table 1
Characterisation of lipids used for preparation of the emulsion and liposome systems.

	Crude herring oil	Polished herring oil	Herring roe phospholipids
PV (mmol LOOH/kg)	5.0 ± 0.1	4.3 ± 0.1	42.7 + 2.1
TBARS (mmol/kg)	0.38 ± 0.02	0.46 ± 0.02	1.08 ± 0.13
Water content (%)	0.351 ± 0.006	0.098 ± 0.003	NA
Lipid classes (%)			
Triacylglycerols	NA	97.6	nd
Cholesterol	NA	0.2	2.1
Unspecified	NA	0.8	0.03
Phospholipids	NA	1.4	97.9
of which PC	NA	NA	84.4
Lyso PC	NA	NA	0.5
PE	NA	NA	14.1
Lyso PE	NA	NA	0.6
Unspecified	NA	NA	0.4
Fatty acid profile (%)			
Saturated	NA	22.4	27.8
Mono-unsaturated	NA	58.8	16.1
of which CET	NA	38.3	3.0
ERU	NA	2.2	0.5
GAD	NA	25.9	9.5
OLA	NA	19.8	36.6
Di-unsaturated	NA	1.7	1.0
Poly-unsaturated[a]	NA	16.0	54.5
of which EPA	NA	40.5	23.4
DHA	NA	40.0	69.0
DPA	NA	4.3	2.0

NA = not analysed; nd = not detected; PC = phosphatidylcholine; PE = phosphatidylethanolamine; CET = cetoleic acid; ERU = erucic acid; GAD = gadoleic acid; OLA = oleic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; DPA, docosapentaenoic acid.

[a] $C > 18$, double bond $\geqslant 3$; the values are given as mean values ± standard error of n measurements or as mean values with maximum coefficient of variation (specified in Section 2 for respective analyses).

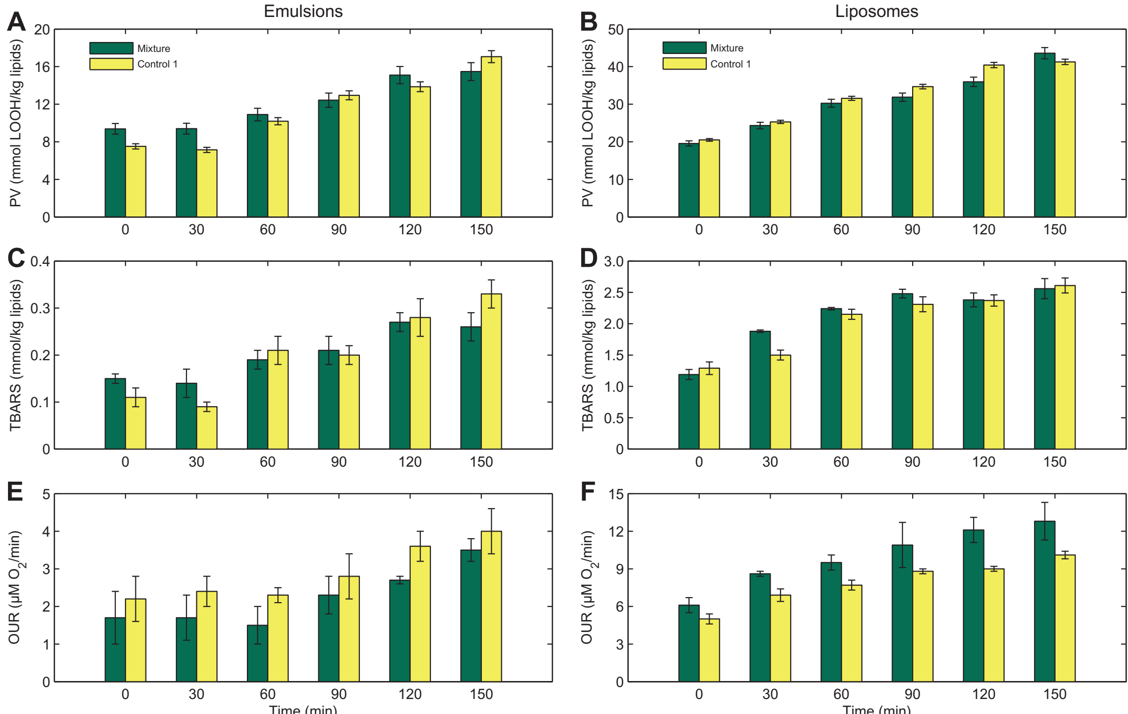

Fig. 2. Development of PV (graphs A and B), TBARS (graphs C and D), and OUR (graphs E and F) in the liposome system (graphs A, C and E), and the emulsion system (graphs B, D and F) during a 2.5 h incubation period. Bars for GJ–lipid blends (Mixture) have dark colour and bars for blends, in which gastric juice was substituted with 0.11 M HCl (Control 1), have light colour. The values are given as average values ± standard error ($n = 5$).

assortment of ultra fresh rest raw material consisting of heads, guts, and muscle trimmings including bones and skins. The crude oil was further polished in order to remove water soluble and polar compounds, inherent moisture, and insoluble impurities which remained in the oil after the production. The compositional characteristics of the lipid substrates are summarised in Table 1.

The peroxide value of the crude oil decreased significantly after washing, from initial 5.0 ± 0.1 mmol LOOH/kg to 4.3 ± 0.1 mmol LOOH/kg, which may be due to a partial removal of peroxidised polar lipids (phospholipids, free fatty acids, mono- and diacylglycerols) or decomposition of lipid hydroperoxides during the polishing procedure. The inherent moisture content was reduced by 72% by polishing. The TBARS values were slightly increased in the polished oil. The polishing step did not have any dramatic deteriorating effect on the oil and, besides improving the PV status, also improved some visual characteristics, such as colour and clarity (figures not shown). The isolated phospholipids were found to be oxidised to a much higher degree than the oil (Table 1). The presence of pre-formed lipid hydroperoxides at various levels is a situation likely to occur in food matrices containing vulnerable marine lipids, therefore the phospholipids were not excluded from the experiments.

The hydrochloric acid (HCl) concentration in the gastric juice (GJ) was found to be 0.109 ± 0.002 M, which is a normal value for healthy humans during fasting (Ulleberg et al., 2011). This value corresponds to pH 1.0, which was verified by a pH-electrode.

3.2. Lipid oxidation in the gastric juice–emulsion model

The most common form of lipids in foods is emulsions or micellar structures (Waraho, McClements, & Decker, 2011). 10% (w/v) herring oil emulsion stabilised by herring phospholipids was chosen for studying oxidation in the gastric juice environment. This is a multiphase food-related system containing marine lipids, which is simple enough to enable interpretation of the measured data. Three different lipid oxidation markers, PV, TBARS and oxygen uptake, were measured to characterise the development of lipid oxidation (Fig. 1A).

The development of peroxide value in the GJ-emulsion system during a 2.5 h incubation period is shown in Fig. 2A. Regardless whether gastric juice was present (Mixture) or substituted with HCl solution (Control 1), the initial peroxide values were doubled and were significantly higher at the end of the incubation period; the trend was consistent for both the Mixture and Control 1. The peroxide level remained unchanged for the first 30 min of incubation, and from 60 min to 150 min showed a linear increase. The initial PV for the Mixture (9.4 ± 0.6 mmol LOOH/kg) was slightly higher than the theoretically expected value (7.8 mmol LOOH/kg – calculated from the PVs determined in the lipid substrates (Table 1)) while the initial PV for Control 1 was consistent with the theoretical value. Preparation of the final experimental mixture might have caused this increase in inherent PV level. The significant increase in PV during the incubation period clearly shows that oxidation of marine lipids in emulsion does occur both in the HCl solution and the authentic human gastric juice. The data did not show a difference between the two environments, and gastric juice therefore seems a prooxidative medium in respect to its acidity rather than its composition.

An initial half an hour "lag" phase during the incubation of the emulsions was apparent, after which the PV steadily increased. The rate of peroxidation in the emulsion during the initial half an hour

might not have been developed to such a degree that an increase in peroxide value could be immediately measured, at least not by the method which was used.

The increase in PV agrees with findings of Lapidot, Granit, & Kanner, 2005b who observed a linear increase in peroxide formation in grilled red turkey muscle during 180 min incubation at 37 °C in simulated gastric juice.

The development of TBARS in the GJ–emulsion system is shown in Fig. 2C. In both the Mixture and Control 1 consistently, the TBARS concentrations remained constant for the first 30 min of the incubation period, followed by a linear increase until the end of the incubation period. The values were significantly higher at the end of the incubation period than at $t = 0$ min, showing occurrence of lipid oxidation in acidic environment. The trend in the TBARS development was the same as the trend in the PV development.

The measured initial values (0.15 ± 0.01 mmol/kg for the Mixture and 0.11 ± 0.02 mmol/kg for the Control 1) substantially deviate from the theoretically expected value (0.52 mmol/kg – calculated based on TBARS determined in the lipid substrates (Table 1)). This discrepancy cannot be satisfactorily explained at this moment. One possible explanation could lie in the methodology. The isolated phospholipids and the polished oil (bulk lipids) were measured with a method which uses a TEP-standard curve for TBARS quantification (Ke & Woyewoda, 1979), while the concentration of TBARS in emulsion/liposomes (multiphase systems) were calculated using a given extinction coefficient (McDonald & Hultin, 1987).

Shortly after diluting the freshly made emulsion with gastric juice or HCl solution, creaming of the emulsions occurred, which could be attributed to a rapid drop in pH which affects the droplet surface charge. The droplets become less negative and therefore less repellent towards each other. Taking representative aliquots from the samples was problematic, which can be seen on the relatively large standard errors. In the study of Hur, Decker, & McClements, 2009 creaming of oil-in-water emulsions was observed after the emulsions passed an *in vitro* digestion model, which was explained by the activity of gastric lipases and by a gradual dilution of the emulsion. Creaming was also observed in the Control 1 measurement with HCl solution. This suggests that creaming was caused mainly by the physicochemical properties of the systems and marginally by the components in the gastric juice, although lipases were expected to be active in the GJ models.

In earlier studies, it was shown that uptake of dissolved oxygen relates to lipid peroxidation in multiphase systems and that oxygen uptake rate (OUR) relates to the rate of lipid oxidation (Kristinova et al., 2009; Mozuraityte, Rustad, & Storro, 2008; Mozuraityte et al., 2006). The OUR development in the GJ–emulsion system is shown in Fig. 2E. The initial oxygen uptake remained unchanged for the first 90 min of the incubation period, followed by a gradual increase reaching double the initial values both in the Mixture and Control 1 at the end of the incubation period.

To verify whether the oxidation is mediated by endogenous metals, presumably iron ions, a strong metal chelator (25 µl of 1 mM EDTA) was added to the Control 1 in the liposome system at the end of the experiment. The background OUR was instantly reduced by 56 ± 2%, indicating presence of endogenous iron in the system. The concentration of the endogenous iron ions was then estimated by approximation from oxygen uptake rates measured for different Fe^{2+} concentrations (data not shown). The level was estimated 17 mg/kg PL (ppm). Since the isolated phospholipids were used as an emulsifier in the emulsions, endogenous iron is inevitably expected also in the emulsions. It is reasonable to assume that oxidation in the liposomes and emulsions was mediated by the endogenous iron. The source of iron contamination could have been steel equipment used during isolation of PL and preparation of liposomes/emulsions.

Although the trend in OUR development correlates well with the PV and TBARS measurements, a constant OUR in the period where PV increased linearly was expected, assuming that lipid oxidation is entirely facilitated by red-ox cycling of iron ions with a steady consumption of oxygen in the red-ox cycle (Mozuraityte et al., 2008). Increasing OUR alongside the linear increase in PV concentration (Fig. 2) suggests partial decomposition of formed lipid hydroperoxides into secondary oxidation products other than TBARS, or non-iron-catalysed peroxidation occurring alongside the iron-mediated oxidation.

Consistently with the outcomes from the PV and TBARS determinations, also the oxygen uptake assay shows that the acidic gastric juice environment does not prevent fatty acids from being oxidised.

3.3. Lipid oxidation in the gastric juice–liposome model

Liposomes are a convenient model system for oxidation studies due to their compositional and structural simplicity, homogeneity and physical stability (Henna Lu, Nielsen, Timm-Heinrich, & Jacobsen, 2011; Mozuraityte et al., 2008). Liposomes are scarce in food matrices and their potential lies mainly in pharmacology as a drug delivery system. Nevertheless, liposomes composed of phospholipids can be related to cell membranes in lean mammal and fish muscles. Therefore the oxidation was followed in 2.5% marine liposomes as well.

The development of the peroxide value in the GJ–liposome system is shown in Fig. 2B. The concentration of peroxides in the liposome system increased linearly during the whole incubation period, at the end of which the initial values were doubled. This linear trend in peroxides formation was consistent and clear for both the Mixture and Control 1 with slopes 0.15 ± 0.01 mmol LOOH/kg min and 0.14 ± 0.01 mmol LOOH/kg min, respectively. No significant difference in the slopes was found between the two systems. No initial lag phase, but a steady increase in PV from $t = 0$ min was observed, showing instant susceptibility of liposomes to oxidation. Liposomes are hollow nm-sized particles with a relatively large total surface area, the cavity is filled with the surrounding aqueous medium containing dissolved oxygen. Oxidation of fatty acids takes place entirely in the interphase, which is formed by a bilayer of phospholipids, where both the outer and inner layer is exposed to the aqueous surroundings. This could explain the high susceptibility to oxidation and absence of a lag phase. In addition, the concentration of endogenous iron coming from PL is higher in the liposomes than in the emulsion due to a higher proportion of PL in liposomes. The initial PV in the Mixture and Control 1 were half the value determined in the isolated PL. One scenario explaining this discrepancy could be that a proportion of existing lipid hydroperoxides was reacted into a variety of secondary oxidation products during preparation of the liposomes, possibly during the sonication step which involves a high energy input.

The development of TBARS in the GJ–liposome system is shown in Fig. 2D. The TBARS concentrations increased both in the gastric juice Mixture and the Control 1. The fastest increase occurred during the first 90 min, followed by a stable phase lasting until the end of the incubation period. The initial TBARS values were doubled at the end of the incubation period for both the Mixture and Control 1. The initial values ($t = 0$ min) for both the Mixture and Control 1 were not significantly different from the TBARS determined in the isolated phospholipids.

Neither PV nor TBARS showed any difference between oxidation in the liposome system containing genuine gastric juice and a system containing simple HCl solution. No difference between GJ and HCl environment was also found for the emulsion system. Lipid

oxidation in the acidic environment seems therefore mainly governed by the acidity of the gastric juice.

The course of oxygen uptake rate during the 2.5 h incubation period in the liposome system is shown in Fig. 2F. At the end of the incubation period the OUR reached double the initial values. The development in the OUR showed no difference between the system with gastric juice and the system lacking gastric juice components apart from HCl. A presence of endogenous iron was found positive in liposomes (explained in Section 3.2). Due to a linear trend in PV formation (Fig 2B) resulting from red-ox cycling of endogenous iron in lipid peroxidation, a constant OUR was expected for the whole oxidation period. The data measured for liposomes also suggest that not all the radicals responsible for binding O_2, i.e. its consumption, might be coming from the steady red-ox cycling of iron, but radicals generated in additional reactions, such as autoxidation, which might be occurring simultaneously, could be involved as well and increase the rate of oxygen uptake.

The initial background OUR in the liposomes was relatively high which required re-saturation steps during oxygen uptake recording (described in Section 2.20). A supply of oxygen into the stomach content is likely to be facilitated by food, drinks and swallowed air during eating (Kanner & Lapidot, 2001). Therefore the PV and TBARS experiments were designed not to be limited by supply of oxygen and the re-saturation steps could be included into the recordings of oxygen uptake.

Consistently with the PV and TBARS measurements, the recordings of oxygen uptake in the liposomes showed occurrence of lipid oxidation in the gastric juice/acidic environment.

3.4. Relation between PV, TBARS and OUR

In liposomes a steadily developing oxidation from the beginning of the incubation period was apparent, while in the emulsions a lag phase during the first 30 min occurred followed by an increase in oxidation (Fig. 2). The data show that in the acidic environment liposomes oxidised more readily and faster than emulsions. Liposome spheres made of phospholipids have smaller diameter (nm-scale) than emulsion particles (μm-scale), which makes the interfacial area larger for liposomes. In addition, the inner cavity of liposomes is filled up with aqueous solution exposing basically all the phospholipids to the aqueous phase containing dissolved oxygen and even enlarging the interfacial area of liposomes. The amount of phospholipids creating the overall interface was slightly higher for liposomes than for emulsions in 12 ml experimental volumes and the concentration of endogenous iron associated with PL was therefore also higher in liposomes. This can explain the higher susceptibility to oxidation of liposomes comported to fat droplets in emulsions.

These findings could have implication for development of delivery systems of marine lipids. Phospholipids are believed to be easier absorbed by the gastrointestinal tract than triacylglycerols (Ramírez, Amate, & Gil, 2001) and are therefore an attractive form of omega-3, and at the same time liposomes are a convenient form for oral supplementation. As shown in this study, marine phospholipids organised as liposomes have the tendency to oxidise under the stomach conditions, which may compromise the positive sides of this delivery system.

The increase in PV during the 2.5 h incubation period was 7 mmol LOOH/kg in the emulsion model and 25 mmol LOOH/kg in the liposome model. The extent of oxidation (under the conditions in this study) therefore is relatively low. Nevertheless, the measurements indicate that PV levels might increase during post-prandial oxidation reaching levels associated with rancidity.

Since primary oxidation products – lipid hydroperoxides (LOOH) – are formed during the incubation period (Fig. 2AB), formation of secondary oxidation product is then expected, assuming that decomposition of the freshly formed lipid hydroperoxides oc-

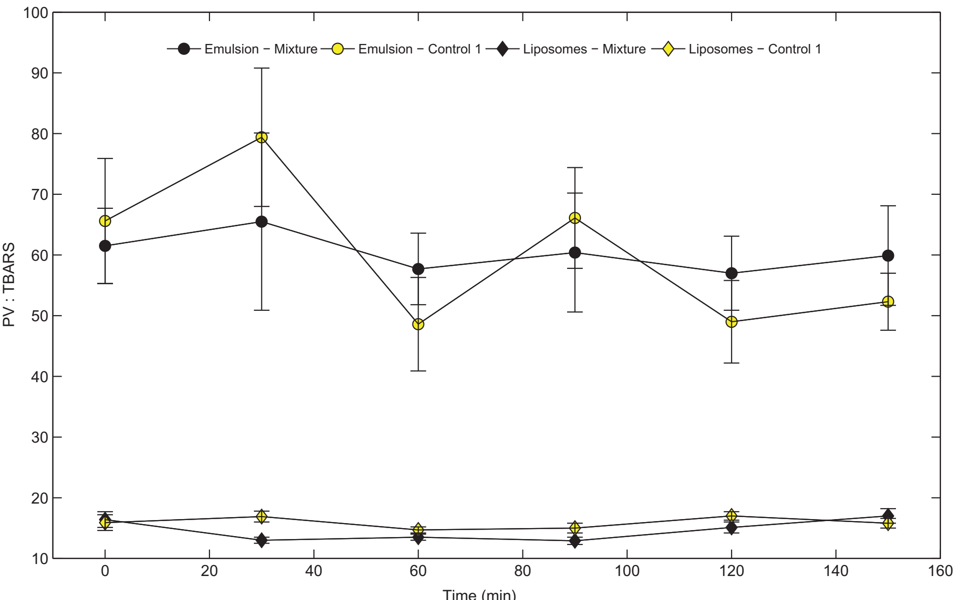

Fig. 3. Ratio between PV and TBARS values in the gastric juice–liposomes (dots) and gastric juice–emulsion (diamonds) systems during a 2.5 h incubation period. Symbols for systems with gastric juice (Mixture) have dark colour, and systems, in which gastric juice was substituted with 0.11 M HCl (Control 1), have light colour. The values are given as average values ± standard error (n = 5).

curs to a certain degree simultaneously. To see how TBARS levels relate to the concentrations of LOOH, a ratio between PV and TBARS levels was plotted for the whole incubation period (Fig. 3). In the emulsion models the concentration of peroxides was 60 ± 3 (Mixture) and 60 ± 11 (Control 1) times higher than the concentration of TBARS throughout the incubation period. In both cases, the production of PV and TBARS appeared simultaneous and was somewhat balanced during the duration of the experiments. The TBARS however represent only a small fraction of secondary oxidation products generated by decomposition of lipid hydroperoxides, therefore this relationship does not apply generally on secondary oxidation products.

The formation of primary and secondary oxidation products during incubation of lipids in the gastric juice is in agreement with outcomes of a number of studies addressing post-prandial oxidation of lipids in a simulated gastrointestinal tract (Kanner & Lapidot, 2001; Lapidot et al., 2005a; Lorrain, Dangles, Genot, & Dufour, 2009). In the study of Staprans et al., 1994 the formation of both lipid hydroperoxides and TBARS in cooked meat increased 15-fold and 7-fold, respectively, during a 3 h incubation in a simulated gastric fluid at 37 °C and pH 3.0. The oxidation was completely inhibited by adding BHT (0.2% lipid weight) and grape seed extract (1% meat weight).

Measurement of oxygen uptake rate gave complementary data to PV and TBARS for both the liposome and the emulsion system – an increasing trend in the rate of oxygen consumption was observed during the 2.5 h incubation period. The increase was more clearly seen in the liposome system, which had relatively high initial OUR and higher concentrations of inherent peroxides and TBARS. A presence of endogenous iron was verified in liposomes. According to theory, formation of one hydroperoxide molecule in the propagation step of iron-mediated oxidation is accompanied by binding one O_2 molecule to an unsaturated fatty acid (Kamal-Eldin, 2003). Such progress then leads to a linear increase in peroxide formation and constant OUR. Assuming that decomposition of the formed peroxides would be in a balance with peroxide formation, but occurring to a lower degree, still a constant rate of oxygen consumption would be expected. No constant OUR was measured for the periods where PV showed a linear increase. Instead, the OUR trend was the same as the trends in PV and TBARS measurements. Decomposition of lipid hydroperoxides into other secondary products than TBARS and additional radical reactions not connected to red-ox cycling of iron could explain the increasing trend in OUR.

3.5. Effect of added compounds

Food ingredients frequently and almost inevitably contain promoters of lipid oxidation in trace or significant concentrations – transition metals (iron, copper) in particular (Martınez-Navarrete, Camacho, Martínez-Lahuerta, Martínez-Monzó, & Fito, 2002), which was also demonstrated on isolated phospholipids. Iron can be present as elemental, ferrous and ferric, or bound within various

Table 2
Net oxygen uptake rates (OUR) for lipid oxidation mediated by iron ions and methemoglobin (metHb) in the gastric juice (GJ)–liposome system and the GJ–emulsion system (Mixtures) and their corresponding controls (Control 1), in which gastric juice was substituted with 0.11 M hydrochloric acid (HCl) solution.

	Liposomes		Emulsion	
	Mixture	Control 1	Mixture	Control 1
	OUR (μM O_2/min)[a]		OUR (μM O_2/min)[a]	
10 μM Fe^{2+}	6.9 ± 0.4	14.8 ± 1.8	2.3 ± 0.3	4.4 ± 0.5
10 μM Fe^{3+}	NA	NA	1.0 ± 0.1	3.5 ± 0.4
0.15 μM metHb	10.3 ± 4.4	10.2 ± 1.0	3.5 ± 0.5	4.4 ± 0.9

[a] Average value ± standard deviation (n = 3); NA = not analysed.

proteins and pigments, such as myoglobin, hemoglobin and enzymes.

In this study, iron ions (Fe^{2+} and Fe^{3+}) and bovine methemoglobin (metHb) were tested in the gastric juice-lipid systems in order to characterise their effect on lipid oxidation in the *in vitro* gastric juice environment. The oxygen uptake method was chosen for evaluation of the effects of these compounds, because the impact of the compounds can be directly seen on changes in the oxygen uptake curves.

The net OUR in the lipid systems with addition of 10 μM Fe^{2+}, 10 μM Fe^{3+} and 0.15 μM metHb is shown in Table 2. Iron activity in both the liposome and emulsion system was significantly reduced, by a factor two, in the gastric juice environment compared to the HCl environment. Thus gastric juice acted as an antioxidant in Fe-mediated oxidation. According to Davis, Multani, Cepurneek, and Saltman (1969), gastric juice has the ability to chelate dietary iron by gastroferrin – a high molecular weight protein secreted into the gastric juice regulating the extent of gastrointestinal iron absorption. This protein has capacity to bind up to 15 mg of iron present in a typical daily diet (Davis et al., 1969). The reduction of the iron-mediated lipid oxidation could be attributed to chelating abilities of inherent gastroferrin. Its concentration and binding constant at pH 4 was not known. It is confusing why endogenous iron coming from phospholipids was not totally chelated by the gastric juice. No clear explanation has been found for this phenomenon. The positively charged endogenous iron ions could be strongly attracted and somewhat associated with the negative polar heads of phospholipids and in competition to chelation by gastroferrin.

MetHb addition to the lipid systems instantly triggered peroxidation. No significant differences between metHb-mediated lipid oxidation in the presence or absence of GJ components apart from HCl suggest that gastric juice does not have the potential to deactivate prooxidative properties of metHb and that heme–iron is active in an acidic environment. When the same concentrations of iron (10 μM) and metHb (0.16 μM) were added to the liposomes and emulsions, the magnitude of the pro-oxidant effects was different in the two systems (Table 2). This could be attributed to different properties of the systems, such as surface area, droplet structure, and the initial levels of lipid hydroperoxides. The data also show that iron embedded in the hem-structure of metHb is a stronger prooxidant than low molecular weight iron. The different nature of the pro-oxidants was also seen on oxygen uptake curves. MetHb-mediated oxidation gave an exponentially decreasing curve while Fe-mediated oxidation proceeded linearly. Linear oxygen uptake in iron-mediated lipid oxidation was explained in the study of Mozuraityte et al., 2008 by red-ox cycling of iron upon production of lipid hydroperoxides.

Beverages, such as wine and tea, show good antioxidant activities *in vitro* due to content of hydroxylated phenolic and polyphenolic compounds (Ghiselli, Nardini, Baldi, & Scaccini, 1998; Ho, Chen, Shi, Zhang, & Rosen, 1992). Therefore selection of five common beverages – orange and berry juice, black coffee, green tea and read wine – caffeic acid (a hydroxylated phenolic compound) and ascorbic acid (Vitamin C) were added individually to the GJ–lipid models in order to evaluate their effect on lipid oxidation in gastric juice. Ascorbic acid was included due to its abundance in fruit beverages. The main interest was to see if the beverages/compounds have the potential to inhibit post-prandial oxidation and therefore only a selected dose corresponding to a small glass (75 ml) was tested.

A group of beverages/compounds showing a pro-oxidative tendency and a group showing antioxidative tendency was distinguished. The % increase of background OUR by the prooxidative group (iron, metHb, ascorbic acid, orange juice and black coffee) in the emulsions and liposomes is shown in Fig. 4A and B, respec-

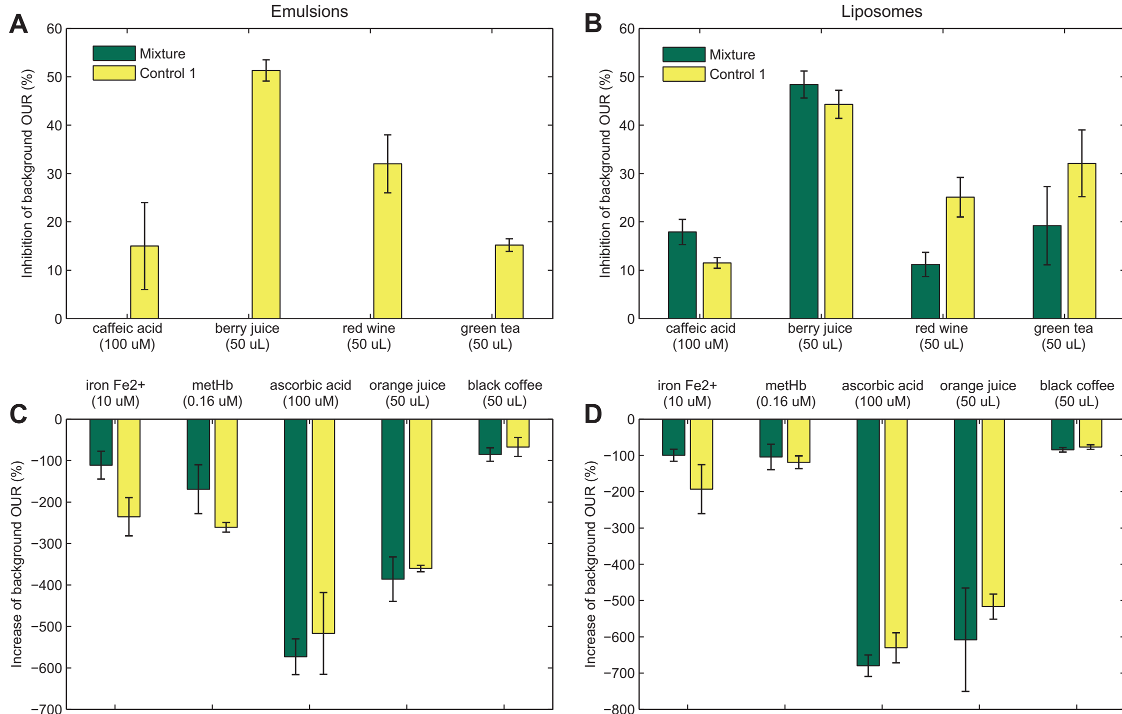

Fig. 4. Acceleration of the background oxygen uptake rate (%) by beverages/compounds in the (A) liposome and (B) emulsion system, and inhibition of the background oxygen uptake rate (%) by beverages/compounds in the (C) liposome and (D) emulsion system. Bars for GJ–lipid blends (Mixture) have dark colour and bars for blends, in which gastric juice was substituted with 0.11 M HCl (Control 1), have light colour. The values are given as average values ± standard error (*n* = 3).

tively. The % inhibition of background OUR by the antioxidative group (caffeic acid, berry juice, red wine and green tea) in the two systems is shown in Fig. 4C and D. Inhibition of the background OUR in the freshly made emulsions was uncertain due to a low background OUR (<3 μM O_2/min). In order to increase the background OUR, the emulsions were allowed to stand for 24 h at room temperature. The lipid phase in the emulsions containing gastric juice however separated from the aqueous phase during the prolonged storage, which made representative oxygen uptake recordings not feasible. Therefore the dataset for GJ–emulsion system (Mixture) is not given in Fig. 4D. In the GJ–HCl system (Control 1) only creaming occurred and the emulsion could be restored by gentle mixing. Enzymes in the gastric juice, such as lipases, could aid phase separation and degradation of the emulsion under the prolonged storage period. No phase separation was observed during the incubation period, but the activity of lipases was expected to some degree in the systems with authentic GJ. The same tendencies in the development of oxidation markers for GJ and HCl systems however indicate that lipid hydrolysis did not have an impact on oxidation of fatty acids during the incubation period.

In both the liposomes (fresh) and the emulsions (aged), the composite berry juice exhibited the best inhibitory effect, followed by green tea, red wine, and caffeic acid, while black coffee, orange juice and ascorbic acid were strongly pro-oxidative and increased the consumption of oxygen by up to 700%.

Increasing the dose of the red wine (1:10, v/v) corresponding to one glass of wine (150 ml) led to an improved antioxidant effect (Fig. 5). A range of flavonoids and phenolic acids are responsible for antioxidative properties of red wine (Ghiselli et al., 1998). Other

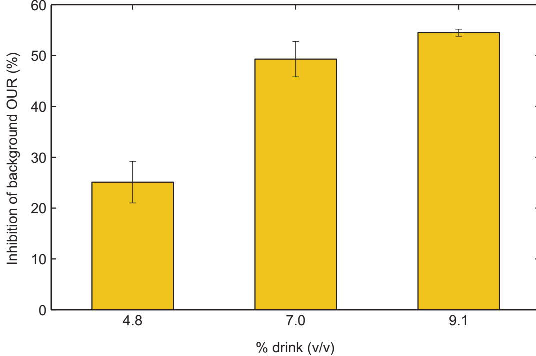

Fig. 5. Inhibition of the background oxygen uptake rate (OUR) (%) by red wine in the HCl–emulsion system. The values are given as average values ± standard error (*n* = 3).

studies also reported a positive effect of red wine on post-prandial oxidation: red wine has shown an inhibition of lipid oxidation of cooked dark turkey meat in a simulated stomach model system (Kanner & Lapidot, 2001), and drinking red wine with three meals of cooked turkey cutlets (compared to drinking water) suppressed malondialdehyde formation in human plasma by 75% (Gorelik, Ligumsky, Kohen, & Kanner, 2008a). According to Argyri, Komaitis, & Kapsokefalou, 2006, interactions of red wine with dietary iron in the stomach may decrease the antioxidant activity of red wine.

Green tea showed a similar antioxidant effect to red wine (Fig. 4). Catechins are the principal antioxidants in green tea (Ho et al., 1992). The outcomes are in agreement with other studies which reported largely positive effects of green tea in *in vitro* multiphase systems: in low density lipoproteins (LDL) antioxidants in green tea hindered peroxidation by radical scavenging mechanism (Salah et al., 1995); green tea extracts showed an antioxidant activity in emulsions and liposomes, unless copper was added into the aqueous phase (Frankel, Huang, & Aeschbach, 1997) and in a study of Serafini, Laranjinha, Almeida, & Maiani, 2000, red wine and green tea were efficient in protecting LDL from oxidation driven by peroxyl and ferryl radicals.

Caffeic acid showed an antioxidant effect in this study. In the study of Kristinova et al., 2009, caffeic acid was found to be strongly prooxidative in liposomes in the presence of iron ions at acidic pH. The prooxidant effect was however observed only for excess of iron in relation to concentration of caffeic acid. This is not the case in the gastric juice models, where caffeic acid was added in excess relative to the endogenous iron concentration.

The principal antioxidants in berry juice are anthocyanins (Zafra-Stone et al., 2007), and in black coffee phenolic acids in cooperation with melanoidins – products of Maillard reactions (Delgado-Andrade & Morales, 2005). Since the concentration of phenolics was reported to be much higher in food than in plasma, post-prandial oxidation in the gastrointestinal tract is believed to be the *in vivo* event where phenolics coming with the food and beverages could protect the unsaturated lipids from oxidation, closely before the lipids are absorbed. These dietary antioxidants can act both as lipid radical scavengers and metal chelators, breaking the propagation of lipid peroxides, and thus enabling the PUFA to exert health benefits (Kerem et al., 2006).

Orange juice and ascorbic acid showed pro-oxidant effects in the GJ–lipid models. Ascorbic acid was abundant in the orange juice (approx. 300 mg/l according to a content list on the packaging). Ascorbate is known to promote lipid oxidation in multiphase systems by the ability to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions (Kanner, Mendel, & Budowski, 1977). Fe^{2+} readily reacts with lipid hydroperoxides being reduced to Fe^{3+}, which is then converted back to Fe^{2+} by another LOOH molecule or ascorbate. Endogenous iron was verified in the phospholipids, which could explain the rapid acceleration of oxidation after addition of both ascorbic acid and orange juice.

The awareness of prooxidative cooperation between ascorbate and low molecular weight iron might be useful for guidance on consumption of vitamin/mineral supplements and marine omega-3 supplements alongside.

In the present study, marine fatty acids in liposomes and emulsions oxidised during 2.5 h incubation at pH 4.0 at 37 °C, regardless whether the environment contained genuine and compositionally complex authentic human gastric juice or simple aqueous HCl solution. No difference in oxidation markers was observed between the same system, which either contained gastric juice or contained HCl solution. Therefore oxidation of emulsified lipids seems to be not aided by the gastric juice components, and the low pH in the stomach appears a favourable medium for oxidation processes. Oxygen uptake rate measurements gave deeper insight into the antioxidative properties of authentic gastric juice. At the experimental conditions, gastric juice was able to partially hinder low molecular weight iron from mediating oxidation and in this way gastric juice acted as an antioxidant. No antioxidant effect on met-hemoglobin mediated oxidation was found. Beverages rich in antioxidants – composite berry juice, green tea, black coffee, and red wine – and caffeic acid significantly reduced oxidation in acidic environment while orange juice and ascorbic acid increased lipid oxidation. The results show that beverages accompanying marine dish have the potential to influence the development of post-prandial lipid oxidation.

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PAPER V

Activity of caffeic acid in different fish lipid matrices: A review

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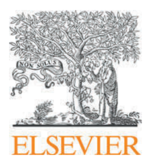

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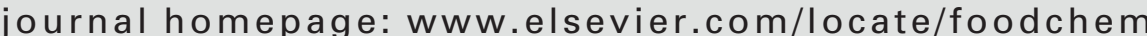

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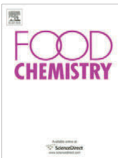

Review

Activity of caffeic acid in different fish lipid matrices: A review

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A B S T R A C T

Caffeic acid, a hydroxycinnamic acid common in different vegetable sources, has been employed as a natural antioxidant for inhibiting oxidation of fish lipids present in different food matrices. The aim of this review is to discuss the mechanisms involved in the antioxidative and prooxidative effects of caffeic acid found in different model systems containing fish lipids. These model systems include bulk fish oils, liposomes from cod roe phospholipids, fish oil emulsions, washed cod mince, regular horse mackerel mince and a fish oil fortified fitness bar. The data reported show that the antioxidant activity depends on the physical state of the lipids and the composition of the intrinsic matrix in which they are situated. Caffeic acid significantly prevented rancidity in both unwashed and washed fish mince, the latter which was fortified with haemoglobin. In the unwashed mince, the activity was however clearly dependent on the lipid to antioxidant ratio. In these systems, an important redox cycle between caffeic acid and the endogenous reducing agents ascorbic acid and tocopherol were further thought to play an important role for the protective effects. The effect of caffeic acid was also highly dependent on the storage temperature, showing higher effectiveness above than below 0 °C. Caffeic acid was not able to inhibit oxidation of bulk fish oils, fish oil in water emulsions and the fish-oil enriched fitness bar. In the liposome system, caffeic acid inhibited haemoglobin (Hb)-promoted oxidation but strongly mediated Fe^{2+} mediated oxidation. In conclusion, caffeic acid can significantly prevent Hb-mediated oxidation in fish muscle foods but its activity in food emulsions and liposomes is highly dependent on the pH, the emulsifier used and the prooxidants present.

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1. Introduction

During the last two decades, seafood products and products rich in $n-3$ polyunsaturated fatty acids (PUFA) have received increasing attention due to their beneficial effects on human health, which can be attributed to the biological properties of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Lee & Lip, 2003). These findings have influenced the increasing demand for $n-3$ PUFA rich fish oils and foods enriched by these oils. However, the storage and processing of fish muscle-based foods or fish oil-containing products are still challenging due to the high susceptibility of PUFA to lipid oxidation (Hultin, 1994). Some studies published during the later years have demonstrated the efficacy of single and combined treatments with natural antioxidants in the inhibition of lipid oxidation of fish oils and fish muscle (Fagbenro & Jauncey, 1994; Pazos, Alonso, Fernández-Bolaños, Torres, & Medina, 2006; Pérez-Mateos, Lanier, & Boyd, 2005; Shahidi & Naczk, 1995; Tang, Sheehan, Buckley, Morrissey, & Kerry, 2001). Together with the ongoing debate about the use of synthetic food additives, such results have lead to the inclusion of plant extracts as antioxidants in fish-oil containing food, and this has became a common practice in the last years.

The natural antioxidants that have gained particular interest as food antioxidants are hydroxycinnamic acids, which are widely distributed and common in seeds, fruits, tubers and the herbaceous parts of many vegetable species (Bravo, 1998). They occur naturally in combination with other compounds usually in the form of esters. Like other phenolic compounds, they can directly trap free radicals or scavenge them through a series of coupled reactions with antioxidant enzymes, and thus delay the onset of lipid oxidation via the decomposition of hydroperoxides (Lewis, 1993). The molecular structure of hydroxycinnamic acids is a key determinant for their radical scavenging and metal chelating properties.

It has been proposed that phenolic compounds that are able to inhibit different prooxidants, cooperate with endogenous antioxidants, and are located on the active oxidation sites will be the most efficient in foods (Frankel, 1998). This implies that identifying how phenolic antioxidants interact with different prooxidants, lipids and antioxidants in fish oil containing foods will be highly valuable in order to protect such foods from rancidity according to natural strategies.

The aim of this work was to review a series of papers where the antioxidant activity exhibited by one of the most active hydroxycinnamic acids, caffeic acid (CaA), have been studied in different marine lipid systems. The large difference between these lipid systems, gives the possibility to discuss the different mechanisms by which caffeic acid can operate as an anti- or prooxidant, in marine foods.

2. Caffeic acid

3,4-Dihydroxycinnamic acid, CaA is a widespread phenolic compound from the group of hydroxycinnamates that is derived biosynthetically from phenylalanine in plants. It occurs naturally in many agricultural products such as fruits, vegetables, wine, olive oil, and coffee (Clifford, 1999). Moreover, the processing of plant foods results in the production of by-products that are rich sources of phenolic compounds (Moure et al., 2001). CaA is present in high proportions in artichoke blanching waters (Llorach, Espín, Tomás-Barberán, & Ferreres, 2002). It is also present in olive mill wastes, which are a major potential source of phenolics considering that the annual production exceeds 7 million tonnes and the medium content of phenolics is approx 1.0–1.8% (Visioli & Galli, 2003).

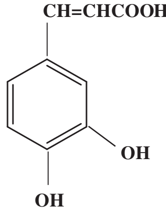


Fig. 1. Molecular structure of caffeic acid.

During the most recent years, CaA and its derivatives have attracted considerable attention due to its various biological and pharmacological activities, including antioxidative activities (Marinova, Yanishlieva, & Toneva, 2006; Nardini et al., 1995; Taubert et al., 2003; Wu et al., 2007). The evaluation of antioxidant capacities requires information on thermodynamics (redox potentials) and kinetic rate constants with different types of radicals, stability of the antioxidant-derived radical, and stoichiometry properties. The molecular structure of CaA (Fig. 1) containing a catechol group with an α,β,-unsaturated carboxylic acid chain is responsible for its efficient interaction with several types of oxidant radicals (Bors, Michel, & Schikora, 1995). The *o*-dihydroxy structure is the natural radical site, producing the *o*-semiquinone after one-electron donation. The lateral double bond conjugated with the catechol group leads to an extensive electron delocalisation, increasing the stability of the *o*-semiquinone radical and, the antioxidant activity (Laranjinha & Lester, 2001). The catechol group also acts as the preferred binding site for trace metals leading to significant chelating activity (Pietta, 2000).

The antioxidant activity of the hydroxycinnamic compounds significantly depends on the structure of the molecules and the microenvironment of the reaction medium (Radtke, Linseisen, & Wolfram, 1998). Different studies comparing the antioxidant effectiveness of hydroxycinnamic and benzoic acids in homogeneous and heterogeneous lipid systems have been carried out, and in these studies CaA has shown a high antioxidant activity (De Leonardis & Macciola, 2003; Gülçin, 2006). CaA is a polar compound with a strong ability for chelating metals (Medina, Gallardo, González, Lois, & Hedges, 2007).

3. Marine lipid oxidation

Lipid oxidation is a rather complex set of reactions where unsaturated fatty acids react with molecular oxygen via a free radical chain mechanism, forming fatty acyl hydroperoxides and non-volatile and volatile hydroperoxide breakdown products. Many of the products, especially the volatile fraction, create undesirable off-flavours known as rancidity resulting in a reduction of the commercial shelf-life of foodstuffs. The lower oxidative stability of unsaturated fatty acids compared to saturated fatty acids is attributed to a lower energy of biallylic and allylic hydrogens compared to that of methylenic hydrogen atoms, and to the high resonance stabilization of the formed radicals (Erikson, 2002). The high proportion of PUFA together with the presence of prooxidants, mainly those containg heme groups are critical factors for development of rancidity in fish muscle and fish oils (Ackman, 1989; Richards & Hultin, 2002). Once the reaction has been initiated, the hydroperoxides formed are converted to free radicals and volatiles. The latter are responsible for the development of rancid off-flavours. The rate and the degree of lipid degradation in fish has been shown to be dependent upon the lipid composition, the presence of prooxidants (e.g. heme, metal ions, pro-oxidative enzymes), the presence of inhibitors (e.g. carotenoids, tocopherol, ascorbate), pH and oxygen concentration, which are all factors which vary throughout the fish/fillet (Undeland, Hall, & Lingnert, 1999). The type and rate of formation of peroxides, saturated and unsaturated aldehydes such

2-pentenal, 2-hexenal, 2,4-heptadienal and 2,4-decadienal, and interaction products resulting from the reaction between carbonyls and proteins as well as amino acids, have been widely reported during oxidative deterioration of species as herring, mackerel, sardines or horse mackerel (Fujimoto, 1993). In addition to PUFA, cholesterol is also modified to oxides and hydroxy derivatives (Saldanha & Bragagnolo, 2007). Lipid peroxidation is not only a quality problem in the edible oil and food industries, but products from lipid oxidation may also have negative health effects. Lipid peroxides increase the tendency of blood to clot by stimulating thrombin generation (Desrumaux et al., 2010) and they may be involved in atherogenesis (Esterbauer, Wäg, & Puhl, 1993). Oxidised cholesterol may be carcinogenic or promote tumour growth (Alexander, 1986). In humans and animals, an inability to control free radical reactions is also associated with premature ageing (Negre-Salvayre, Coatrieux, Ingueneau, & Salvayre, 2006).

Studies from the last ten years have demonstrated that CAa can act as an inhibitor of marine lipid oxidation. The early papers by Banerjee (2006) and Chung, Walker, and Hogstrand (2006) showed the first applications of CAa as antioxidant for marine lipids and proteins. The authors have shown effectiveness for inhibiting the lipoxygenase activity of mackerel muscle when CaA was employed in concentrations ranging 10–100 μM (Banerjee, 2006). Chung et al. (2006) suggested that CAa may protect cultured fish cells against oxidative stress through expression of zinc-induced antioxidant proteins Its activity in fish lipid systems will be discussed in more detail later.

3.1. Effect of lipid oxidation on proteins

Lipid oxidation occurring in fish muscle has an important role in the loss of rheological properties. Lipid and protein interactions can play an important role in the stability and function of muscle proteins. Lipid oxidation products have been demonstrated to affect proteins through the formation of crosslinkages and provoking toughening in late stages of frozen storage of lean species (Soyer & Hultin, 2000). Changes in the protein structures induced by lipid oxidation products and e.g. reactive oxygen species, may thus influence both the texture and the water holding capacity of fish muscle. It is hypothesised that proteins actually oxidise before lipids in muscle tissue since they are in contact with the aqueous phase where radicals like hydroxyl radical are formed (Srinivasan & Hultin, 1995). It could then be the less reactive and long-life protein radicals that mediate the oxidative attack into the hydrophobic interior of membranes. Despite an increasing interest in protein oxidation within the scientific community, it is unclear whether lipid oxidation induces protein oxidation or vice versa. Oxidised proteins with changes in their sulfhydryl groups may be more labile, forming crosslinkages much more rapidly than un-oxidised proteins when subjected to freezing, thawing or a low pH. This may then seriously affect the texture properties of fish muscle.

4. Activity of caffeic acid in different fish lipid containing systems. Main features influencing antioxidant activity

Inhibition of lipid oxidation is important to increase the shelf-life of fish muscle, fish oils and fish oil-containing products during storage and processing. Several factors should be taken into account in the design of antioxidant strategies aimed to minimise rancidity. The nature and characteristics of the lipids, the physical state of the lipids, the food composition and the microenvironment are all well-known factors influencing antioxidant effectiveness.

This work reviews the activity of CaA in different fish lipid systems with increasing complexity: bulk fish oils, liposomes from cod roe phospholipids, fish oil-in water emulsions, washed cod mince and crude horse mackerel mince. The discussion also includes the use of CaA in a commercial fitness bar enriched with fish oil. Table 1 shows the characteristics of the different systems described in this manuscript. The activity of CaA in the different systems has been compared with a less effective hydroxycinnamic acid, coumaric acid, which lacks the second hydroxyl group in the phenol ring (Shahidi & Naczk, 2004). To quantify the effect of CaA in the different systems, the prolongation of the lag phase, the effect on the maximum level of oxidation by-products formed, the effect on the rate of oxidation and the percent of inhibition on the formation of oxidation by-products are described (Figs. 2 and 3). Percent of inhibition was calculated during the propagation period as follows:

$$\%\text{Inhibition} = [(C - S)/C] \times 100,$$

where C = oxidation product formed in control samples and S = oxidation product formed in sample according to Frankel (1998).

4.1. Activity of caffeic acid in bulk fish oil

Bulk fish oils are homogeneous systems in which the affinities of antioxidants towards the air–oil interfaces are determining their effectiveness against lipid oxidation. Hydrophilic antioxidants have been suggested to be more effective against oxidation in bulk oils by being oriented in the air–oil interface whereas in water emulsions they are found in the water phase (Frankel, 1998). Recent data also suggest that the increased efficacy of hydrophilic components in bulk oils could be attributed to their localisation in micellar structures formed by minor components, such as phospholipids, free fatty acids or monoglycerides present in the oil (Chaiyasit, Elias, McClements, & Decker, 2007). Polar antioxidants, which are more readily concentrated at the interface of association colloids than at the oil–air interface, could be more active in bulk oils than non-polar antioxidants (Chaiyasit et al., 2007).

However, CaA showed a low inhibition of oxidation in bulk cod liver oils (Sanchez Alonso, 2002). It was not able to inhibit the formation of conjugated diene and triene hydroperoxides. There was no effect on the prolongation of the lag phase nor on the rate of oxidation. Its activity was lower than the synthetic antioxidants propyl gallate and hydroxytyrosol, the latter, a single *o*-dihydroxy phenol derived from oleuropeine, has been a well recognised antioxidant in bulk oils with similar molecular structure (Pazos, Alonso, Sanchez, & Medina, 2008). The low activity of CaA could be attributed to its limited solubility in the oily oil phase. The partitioning coefficient of CaA expressed as% oil was 0.30 (Medina et al., 2007) and the CaA remained relatively insoluble in the fish oil. Coumaric acid also did not show any efficiency in inhibiting oxidation of fish oils. Marinova, Toneva, and Yanishlieva (2009) have described CaA antioxidant effectiveness during autoxidation of sunflower oil at 100 °C, where the triacylglycerols are more saturated lipids than in fish oils. In addition, the antioxidant carrier can play an important role for determining antioxidant effectiveness of phenolic acids. The use of acetone as solvent for CaA has lead to a significant inhibition of lipid oxidation in cod liver oil subjected to high oxidative conditions, 100 °C and air flow of 20 L/h (De Leonardis & Macciola, 2003). However, most organic solvents are not food grade and therefore the choice of a carrier should be done carefully.

4.2. Heterogeneous fish lipid systems

Lipid oxidation in multicomponent foods is an interfacial phenomenon affecting pro-oxidant and antioxidant constituents depending on the rate of oxygen diffusion and interactions between unsaturated lipids, metal initiators, radical generators and

Table 1
Description of the marine lipid systems employed in this work for illustrating the activity of CaA.

Marine lipid system	Reference	Caffeic acid concentration	pH	Percent of fat and moisture	Addition of prooxidant	Storage conditions (±light, temperature)	Methods used to determine oxidation	Other information
Cod liver oil		100 mg/kg		Fat:100 Water: 0	No	Darkness, 40 °C	Conjugated dienes and trienes	
Liposomes from cod roe Liposomes made from marine (cod roe)	0.018–180 mg/kg Water: 98.5	5.5		Fat: 1.5	Fe^{3+} Hb	Darkness, 30 °C	phospholipids Oxygen consumption	Kristinová et al. (2009) phospholipids
Cod liver oil in water emulsions	Sørensen et al. (2008)	100 mg/kg	3 or 6	Fat: 10 Water: 90	$FeSO_4$	Darkness, 20 °C	ESR, PV, volatiles	Emulsions made with Tween or Citrem as emulsifiers Buffer:
Washed cod mince	Larsson and Undeland (2010)	Ice storage: 10–1000 mg/kg Frozen storage: 10 and 100 mg/kg	6.3	Fat: 0.6 Water: 87	Hb	Darkness. Ice and frozen (−20 °C)	PV, rancid odour, loss of redness, carbonyls, protein salt solubility, vitamin E	
Horse mackerel mince	Medina et al. (2007), Medina et al. (2009)	Ice storage: 10–100 mg/kg Frozen storage: 100 mg/kg	6.7	Fat: 2 Water: 80	No	Darkness, 4 °C, −10 °C and −18 °C	PV, TBARS, volatiles rancid odour, vitamin E	
Fitness bar with fish oil (Maritex 43–01) emulsion	Horn et al. (2009)	75, 150, 300 mg/kg		Fat: 5 Water added: 2.1	No	Darkness, room temperature	PV, volatiles, sensory, FAME, vitamin E	Ingredient: brown sugar syrup honey fig-date mix, wheat flour, rolled oats, Kelloggs rice krispies raisins and apricots Fish oil emulsion made with sodium caseinate

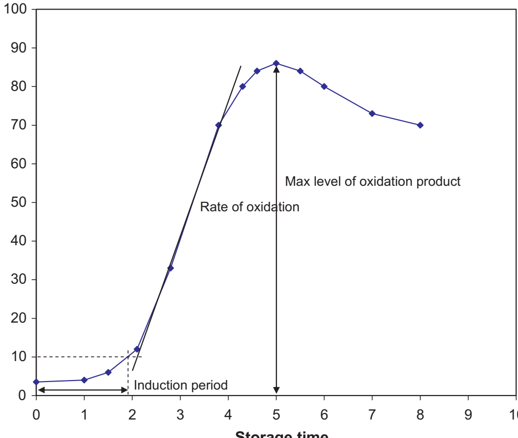


Fig. 2. Graph illustrating formation of oxidation products such as peroxides, TBARS or volatiles in a fish lipid matrix. The calculation of the induction period of oxidation, the rate of oxidation and the maximum level of peroxides, TBARS or volatiles formed are indicated on the graph.

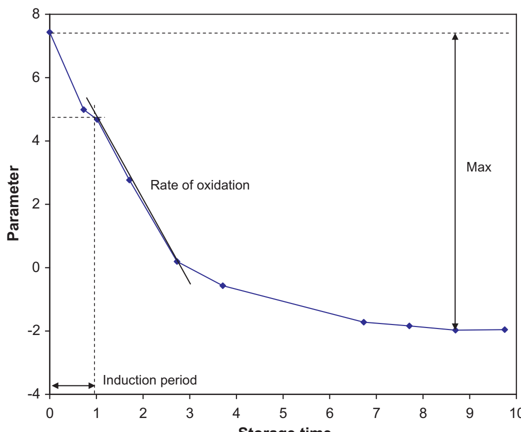
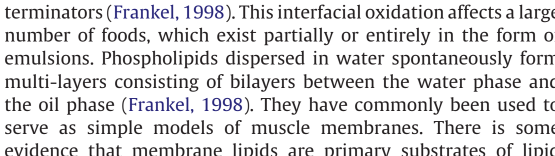


Fig. 3. Representative kinetics of the loss of redness and endogenous α-tocopherol in a fish lipid matrix. The calculation of the induction period of oxidation, the rate of oxidation and the maximum loss of redness or α-tocopherol are indicated on the graph.



terminators (Frankel, 1998). This interfacial oxidation affects a large number of foods, which exist partially or entirely in the form of emulsions. Phospholipids dispersed in water spontaneously form multi-layers consisting of bilayers between the water phase and the oil phase (Frankel, 1998). They have commonly been used to serve as simple models of muscle membranes. There is some evidence that membrane lipids are primary substrates of lipid oxidation, and the interactions of phenolic antioxidants with cell membrane lipid bilayers have lately been used for establishing different interaction mechanisms and different biological activities of antioxidants (Perez-Fons, Aranda, Guillen, Villalain, & Micol, 2006).

A wide range of foods such as mayonnaise, salad dressing and milk are oil-in-water emulsions where oil droplets are dispersed in an aqueous phase. Butter and margarine are examples of

water-in-oil emulsions, where water droplets are dispersed in the continuous oil phase. Emulsification leads to a large increase in the interfacial area and therefore lipid oxidation is a severe problem in emulsions. Lipid oxidation mechanisms in emulsions are quite complex. Hence, lipid oxidation has been shown to be affected by several factors, amongst which the nature of emulsifiers, the pH and the partitioning of antioxidants into the different phases are key factors affecting the reaction rate (Frankel, 1998). Moreover, interactions between the emulsifier, the antioxidant and metal ions present in the emulsion have been suggested to affect the efficacy of antioxidants (Sørensen et al., 2008).

Several antioxidants have been proposed for stabilizing oil emulsions (Frankel, 1998). The hydrophilic and lipophilic characteristics of these compounds largely influence their antioxidant activity. The capacity to establish hydrophobic and/or hydrophilic interactions depending on the environment has also been suggested as a key factor for effectiveness in multicomponent foods. Antioxidants having hydrophobic cores with hydrophilic hydroxyl groups may be exposed to both or any of the two regions, resulting in surfactant-like accumulation and activity in water–oil interfaces (Pazos, Gallardo, Torres, & Medina, 2005).

4.2.1. Activity of caffeic acid in liposomes

Liposomes supplemented with CaA and coumaric acid in the presence of iron or haemoglobin as prooxidants were subjected to oxidation during storage at 30 °C for up to 30 min. Oxidative reaction was followed studying the oxygen uptake rate (Kristinová, Mozuraityte, Storrø, & Rustad, 2009). Results indicated that CaA inhibited haemoglobin-induced oxidation at concentrations from above 0.1 to 1000 μM and was inactive at 0.1 μM CaA. The efficiency had an increasing tendency with increasing CaA concentration. The percent of inhibition ranged between 30% at 1 μM and 50–57% at 300–1000 μM. Coumaric acid did not exhibit any protective activity at the tested concentrations. CaA strongly enhanced iron-induced oxidation at all tested concentrations except for the lowest concentration (0.1 μM), at which it did not have any significant effect on the oxidation rate. The prooxidant effect decreased by using CaA concentrations higher than 200 μM. This prooxidant activity is the result of the ability of CaA to reduce Fe^{3+} via the so called intra-molecular electron transfer at molar ratio ⩾0.1 as discussed by Kristinová et al., 2009. The reaction releases Fe^{2+} contributing to the enhanced lipid oxidation in liposomes. However, CaA did not exhibit any significant effect when iron was in excess. When iron is present in great abundance, a total breakdown of CaA was reported to follow complex formation (Hynes & O'Coinceanainn, 2004). This could explain the markedly lower prooxidative activity at 1 μM CaA concentration.

Antioxidative properties of CaA on Hb-mediated oxidation could be explained by the capability of CaA to form noncovalent bonds to proteins (Rawel, Meidtner, & Kroll, 2005). It could be assumed that CaA in the aqueous phase of the liposome solution can enter the heme crevice of Hb and/or bind to ferrylhemoglobin reducing the oxoferyll moiety to less prooxidative metmyoglobin.

4.2.2. Activity of caffeic acid in emulsions

Table 2 summarises the results obtained by Sørensen et al. (2008) on the supplementation of CaA to fish oil emulsions prepared with two different emulsifiers, Citrem and Tween at two different pH: 3 and 6. Apart from oxidation assessment, interactions between the antioxidant and iron were evaluated by UV-spectrophotometry measurements and by observations of formed nanoparticles by Cryo-TEM. The adsorption of the antioxidant on the emulsifier surface was also determined.

Oxidation in emulsions was found to increase with decreasing pH and in the presence of iron. Tween resulted in faster oxidation than Citrem, despite negative droplet charge of Citrem, which could be expected to attract iron to the surface and thereby increase oxidation. No significant antioxidant activity of CaA was observed in any of the two types of emulsion. Using Citrem as emulsifier, CaA showed strong prooxidant activity at pH 3, and no interactions between CaA and iron were observed. In these emulsions, iron ions are expected to be localised at the negatively charged droplet surface. Thus, iron is expected to exist as free metal ions capable of reacting with the hydroperoxides to propagate oxidation. Results obtained by Deiana, Gessa, Pilo, Premoli, and Solinas (1995) and Gülçin (2006) showed that CaA was capable of reducing Fe^{3+} to Fe^{2+}, thereby propagating lipid oxidation. Moreover, results obtained by Brenes-Balbuena, Garcia-Garcia, and Garrido-Fernandez (1992) have showed that CaA oxidised in the presence of iron. This might explain the increased lipid oxidation in the presence of CaA when iron was present.

Additionally, in emulsions without iron at pH 3, CaA gave lower levels of peroxides and a significant prooxidative effect on volatiles formation. This finding suggests that CaA may also reduce low levels of endogeneous Fe^{3+} iron present in the fish oil or emulsifier and that endogenous iron in the reduced state (Fe^{2+}) was responsi-

Table 2
Effect of CaA or coumaric acid (100 mg/kg) on lipid oxidation in oil-in-water emulsions with iron added as prooxidant (100 uM) during storage at 20 °C for up to 7 days. The data are related to controls with no addition of phenolic acids. Adapted from Sørensen et al. (2008).

Measure of lipid oxidation	Prolongs lag phase	Effect on the max level of oxidation by-products	Effect on rate of oxidation	Inhibition at day 5 and 7 (%)
Caffeic acid, Citrem as emulsifier, pH 3				
PV	No	Yes, increased max level	Yes, after 2 days rate increased,	−125
Total volatiles	No	Yes increased max level	Yes, rate increased from day 0	−667
Caffeic acid, Citrem as emulsifier, pH 6				
PV	Yes, from 0 to ca. 2 days	Yes, 80% reduction.	Yes, 89% reduction	77
Total volatiles	No	No	No	0
Caffeic acid, Tween as emulsifier, pH 6				
PV	Yes, from 0 to 2 days	Yes, 78% reduction	Yes, 86% reduction	78
Total volatiles	No	Yes, increased max level	Yes, increased max level	−40
Coumaric acid, Citrem as emulsifier, pH 6				
PV	No	No	No	4.5
Total volatiles	No	No	No	16
Coumaric acid, Tween as emulsifier, pH 6				
PV	No	No	No	−2.8
Total volatiles	No	No	No	6

ble for promoting formation of volatiles. CaA could also accelerate the decomposition of hydroperoxides due to its lower redox potential ($E^{\circ}_{ROO} = 1000$ mV;$E^{\circ}_{CaA} = 540$ mV). (Choe & Min, 2005; Hotta et al., 2002).

At pH 6, CaA was able to reduce the amount of peroxides formed in emulsions containing Tween, but increased the formation of volatiles. In Citrem emulsions, CaA reduced the formation of peroxides but had no effect on volatiles. It has been described that at acidic pHs, iron solubility is higher (Aslamkhan, Aslamkhan, & Ahearn, 2002), and therefore iron promoted oxidation is expected to be higher at pH 3 than at pH 6. The latter was observed in the study by Sørensen et al. (2008).

Coumaric acid had no effect on the formation of peroxides and volatiles neither in emulsions using Tween nor Citrem. Zeta potential and UV-measurements did not indicate interactions between iron and phenolic compounds at pH 3. However, at pH 6, both CaA and coumaric acid interacted with iron and this interaction may have resulted in the formation of an iron-polyphenol complex and nanoparticles. It may be speculated that the interaction between iron and the phenols may prevent them from acting as free radical scavengers. However, the finding that the phenolic compounds generally reduced the formation of peroxides and free radicals compared to the control at pH 6 in both Citrem and Tween emulsions fortified with iron suggests that the phenolic compounds still had free radical scavenging activities, despite the observed interaction with iron. In emulsions where iron catalyses peroxide decomposition and thereby decreases peroxide value (PV), a simultaneous increase in volatiles may be expected.

Addition of CaA or coumaric acid decreased peroxide value (PV) without increasing the formation of volatiles in the Citrem emulsions irrespective of the type of phenolic compound, but not in the Tween emulsions in the presence of CaA. Taken together, these findings may suggest that the combined ability of both Citrem and the polyphenols to form complexes with iron prevented iron from decomposing peroxides. Moreover, it also seemed that CaA lost its ability to reduce Fe^{3+} to Fe^{2+} when Citrem was used, but not when Tween was used. This might be due to the metal chelating properties of Citrem.

It was found that neither CaA nor coumaric acid were adsorbed at the oil–water interface (Sørensen et al. (2008). Both are polar compounds which remain mainly in the aqueous phase.

4.2.3. Activity of caffeic acid in fish muscle based food

Fish muscle is a complex material where lipid oxidation depends on several factors. In live fish, the oxidative stability is controlled by the balance between prooxidants and antioxidants (Decker, Livisay, & Zhou, 2000) and by enzymes controlling the reactive oxygen species. However, after death, a chain of post-mortem reactions changes the prooxidant/antioxidant balance of fish muscle. These dynamic conditions render procedures aimed to minimise lipid oxidation difficult since both concentrations and activities of endogenous oxidants can change significantly. Exogenous antioxidants can interact with the endogenous prooxidants and antioxidants, provoking changes in their scavenging or reducing activities.

Amongst the different lipid classes of fish muscle, phospholipids (membrane lipids) which usually represent ~1% of the total lipids are believed to be the primary subtractes of lipid oxidation leading to rancidity (Gandemer & Meynier, 1995). This is because of their high degree of unsaturation, large surface area and the proximity to oxidation catalysts located in the aqueous cell phases. Recent studies have revealed that the presence of an exogenous antioxidant at the location where oxidation is initiated or propagated could be essential for antioxidant efficacy (Caturla, Vera-Samper, Villalain, Mateo, & Micol, 2003). Therefore, it is suggested to direct the antioxidant towards membrane lipids instead of triglycerides for decreasing muscle oxidation. Such directionality seems to be defined by the antioxidant concentration and the polarity of the antioxidant carrier solvent (Raghavan & Hultin, 2005).

4.2.4. Activity of caffeic acid in washed cod mince and minced horse mackerel muscle

Washed fish muscle mince has become a common model for studying lipid oxidation of fish muscle. It has the structure of muscle, i.e., with intact myofibrillar proteins and membranes, but is free of most endogenous triacylglycerols, pro- and antioxidants. An exception is α-tocopherol, which is bound to the cellular membranes. Washed fish mince thus provides an opportunity to study lipid oxidation as a function of controlled physiological levels of prooxidants, antioxidants and lipids at different conditions of pH and moisture (Undeland, Kristinsson, & Hultin, 2004). Table 3 summarises the results found by Larsson and Undeland (2010) following the addition of CaA to Hb-fortified washed cod minced muscle. During ice storage, CaA was found to be an efficient inhibitor of oxidation. CaA inhibited the formation of peroxides, rancid odour and loss of redness completely at a concentration of 50 ppm or more. The threshold was between 15 and 50 ppm. CaA (⩾100 ppm) also inhibited the formation of protein carbonyls and loss of protein salt solubility. No protective effect was observed by 10 ppm CaA on these parameters. Endogenous α-tocopherol was fully protected by 1000 ppm caffeic acid throughout the storage period, while 10 ppm could only decrease initial degradation compared to control.

Coumaric acid (50–200 ppm) was not effective for inhibiting lipid oxidation. There was a trend that increased concentration of coumaric acid decreased the rate of PV formation and also slightly lowered the maximum PV level. The same trend was seen for rancid odour. There was no observed effect on loss of redness by addition of coumaric acid (50–200 ppm) compared to control.

Regarding storage at −20 °C, CaA (10–100 ppm) could not inhibit formation of peroxides, although the maximum level was decreased by ~30% compared to control. It was difficult to find differences in protein carbonyls since there were only quite small increases in these products in all samples with storage time. There was no apparent effect of CaA on protein salt solubility where the effect of freezing overshadowed all possible differences. Further, CaA could not protect endogenous α-tocopherol during storage.

In minced fish muscle the decrease in particle size, the incorporation of oxygen and the loss of decompartmentation can accelerate lipid oxidation. Fish mince is thus an excellent model system to study processing and storage conditions for short storage times. Table 4 shows the effect of CaA and coumaric acid in chilled and frozen horse mackerel mince (Medina, González, Iglesias, & Hedges, 2009; Medina et al., 2007). CaA was highly effective for preventing oxidation of chilled horse mackerel in a concentration dependant manner between 10 and 100 ppm (Medina et al., 2007). CaA ⩾100 ppm, completely inhibited lipid oxidation. CaA >50 ppm also inhibited the loss of endogenous α-tocopherol in a concentration dependant manner. Surprisingly, ascorbic acid, a water soluble endogenous antioxidant present in fish muscle, was rapidly consumed in presence of increasing amounts of CaA (Iglesias, Pazos, Andersen, Skibsted, & Medina, 2009). This consumption did not affect the inhibition of lipid oxidation achieved through the addition of CaA. Coumaric acid was also able to inhibit oxidation in chilled horse mackerel mince but its effect was lower than that of CaA (Medina et al., 2007).

Regarding the effectiveness in frozen fish, the antioxidant efficacy of CaA in chilled samples showed higher inhibition of oxidation than in frozen samples. The effectiveness of CaA measured by the percent of inhibition on the formation of lipid oxidation products formed did not differ largely between storage at −10 and −18 °C.

Table 3
Ice/Frozen storage. Effect of CaA or coumaric acid on lipid oxidation in washed cod mince with haemoglobin (Hb) added as prooxidant (20 μM) during storage at 0°C for up to 10 days in chilled samples and during storage at −20 °C for up to 14 weeks in frozen samples. The data are related to controls with no addition of phenolic acids. Adapted from Larsson and Undeland (2010).

Measure of lipid oxidation	Prolongs lag phase	Effect on max level	Effect on rate	Effect of AUC (area under the curve)
Caffeic acid, 10 ppm, chilled				
PV	Yes, 0,06 days	No	No	9% Reduction
Rancid odour	Yes, 0.5 days	Yes, 12% reduction	No	7% Reduction
Carbonyls	No	No	No	
Solubility	No	No	No	
Redness	Yes, 0.7 days	No	No	
α-Tocopherol	No	No	Yes, 43% reduction	3% Reduction
Caffeic acid, 15 ppm, chilled				
PV	Yes, 1.5 days	Yes, 41% reduction	Yes, 74% reduction	72% Reduction
Rancid odour	Yes, 1.6 days	Yes, 18% reduction	Yes, 25% reduction	20% Reduction
Redness	1.1 days	No	Yes, 69% reduction	
Caffeic acid, 50 ppm, chilled				
PV	Yes, no oxidation	Yes, 99% reduction	Yes, no oxidation	99% Reduction
Rancid odour	Yes, no oxidation	Yes, 98% reduction	Yes, no oxidation	98% Reduction
Redness	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Caffeic acid, 100 ppm, chilled				
PV	Yes, no oxidation	Yes, 99% reduction	Yes, no oxidation	99% Reduction
Rancid odour	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	99% Reduction
Carbonyls	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Solubility	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Redness	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
α-Tocopherol				
Caffeic acid, 200 ppm, chilled				
PV	Yes, no oxidation	Yes, 99% reduction	Yes, no oxidation	99% Reduction
Rancid odour	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	96% Reduction
Redness	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
α-Tocopherol	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Caffeic acid, 1000 ppm, chilled				
PV	Yes, no oxidation	Yes, 99% reduction	Yes, no oxidation	100% Reduction
Rancid odour	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	100% Reduction
Carbonyls	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Solubility	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
Redness	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	
α-Tocopherol	Yes, no oxidation	Yes, no oxidation	Yes, no oxidation	100% reduction
Coumaric acid, 50 ppm, chilled				
PV	Yes, 0.02 days	Yes, 11% reduction	Yes, 13% reduction	12% Reduction
Rancid odour	Yes, 0.07	Yes, 16% reduction	Yes, 30% reduction	12% Reduction
Redness	No	No	No	
Coumaric acid, 100 ppm, chilled				
PV	Yes, 0.03 days	Yes 17% reduction	Yes, 23% reduction	19% reduction
Rancid odour	Yes, 0.10	Yes, 28% reduction	Yes, 39% reduction	25% reduction
Redness	No	No	No	
Coumaric acid, 200 ppm, chilled				
PV	Yes, 0.11 days	Yes 17% reduction	Yes, 74% reduction	37% Reduction
Rancid odour	Yes, 0.20	Yes, 25% reduction	Yes, 56% reduction	25% Reduction
Redness	No	No	No	
Caffeic acid, 10 ppm, Frozen				
PV	Yes, 0.37 weeks	Yes, 28% reduction	No	25% Reduction
Carbonyls	No	No	No	
Solubility	No	No	No	
Redness	Yes, not measured	Yes, 19% reduction	Missing	
α-tocopherol	No	No	Yes, 42% reduction	1% Reduction
Caffeic acid, 100 ppm, Frozen				
PV	Yes, 0.61 weeks	Yes, 34% reduction	Yes, 20% reduction	43% Reduction
Carbonyls	No	No	No	
Solubility	No	No	No	
Redness	Yes, not measured	Yes, 23% reduction	Yes, 51% reduction	
α-Tocopherol	No	No	Yes, 77% reduction	3% Reduction

These results indicated that CaA was a very active antioxidant for horse mackerel mince and retarded both degradation of endogenous α-TOH and propagation of lipid oxidation measured as hydroperoxides, volatiles and TBARS. Its efficacy was highly related to its capacity for donating 12.2 μmol electrons/mg antioxidant and showed increasing effect with increasing CaA addition (10–100 ppm). The effect of CaA on the endogenous reducing agents present in fish muscle provoked a significant protection of the fish muscle stability. A redox recycling reaction amongst CaA, ascorbate and tocopherol has been demonstrated (Iglesias et al., 2009). Degradation of endogenous ascorbate ($AscH^-$) was accelerated at higher concentration of CaA in fish tissue, suggesting a role of $AscH^-$ in regeneration of CaA analogous to the observed regeneration of α-TOH by $AscH^-$ in biological systems. Such reaction is thermody-

Table 4
Effect of CaA or coumaric acid on lipid oxidation in minced horse mackerel muscle (100 uM) during storage at 4 °C for up to 10 days and during frozen storage at −10 °C and −18 °C. The data are related to controls with no addition of phenolic acids. Adapted from Medina et al. (2007) and Medina et al. (2009).

Measure of lipid oxidation	Prolongs lag phase	Effect on max level	Effect on rate	Average inhibition during the propagation phase
Caffeic acid, 10 ppm, chilled				
PV	No	Yes, 75% reduction	Yes, 70% reduction	75% Reduction
TBARS	Yes, from 2 to 4 days	Yes, 66% reduction	Yes, 88% reduction	63% Reduction
a-Tocopherol	Yes	Yes, 17% maintained	Yes, 15% reduction	100% Reduction
Ascorbic acid	Yes	Yes, 27% maintained	No	No
Caffeic acid, 25 ppm, chilled				
PV	Yes, from 0 to 4 days	Yes, 72% reduction	Yes, 70% reduction	77% Reduction
TBARS	Yes, from 0 to 4 days	Yes, 66% reduction	Yes, 90% reduction	59% Reduction
a-Tocopherol	Yes	Yes, 41% maintained	Yes, 47% reduction	100% Reduction
Ascorbic acid	Yes	Yes, 41% maintained	No	No
Caffeic acid, 50 ppm, chilled				
PV	Yes, from 0 to 4 days	Yes, 80% reduction	Yes, 72% reduction	80% Reduction
TBARS	Yes, from 0 to 4 days	Yes, 66% reduction	Yes, 91% reduction	54% Reduction
a-Tocopherol	Yes	Yes, 65% maintained	Yes, 53% reduction	100% Reduction
Ascorbic acid	Yes	Yes, 20% maintained	Yes, −52%	No
Caffeic acid, 100 ppm, chilled				
PV	Yes, from 0 to the end of the experiment	Yes, no oxidation	Yes, no oxidation	99% Reduction
TBARS	Yes, from 0 to the end of the experiment	Yes, no oxidation	Yes, no oxidation	98% Reduction
a-Tocopherol	Yes	Yes, 100% maintained	Yes, no oxidation	100% Reduction
Ascorbic acid	Yes	Yes, 1% maintained	Yes, −93%	No
Caffeic acid, 200 ppm, chilled				
PV	Yes, from 0 to the end of the experiment	Yes, no oxidation	Yes, no oxidation	100% Reduction
TBARS	Yes, from 0 to the end of the experiment	Yes, no oxidation	Yes, no oxidation	100% Reduction
Coumaric acid, 100 ppm, chilled				
PV	Yes, from 0 to 2 days	Yes, 67% reduction	Yes, 40% reduction	70% Reduction
TBARS	Yes, from 0 to 2 days	Yes, 66% reduction	Yes, 68% reduction	55% Reduction
Caffeic acid, 100 ppm, −10 °C				
PV	No	Yes, 30% reduction	Yes, 48% reduction	52% Reduction
TBARS	No	Yes, 27% reduction	Yes, 50% reduction	43% Reduction
Protein solubility	No	No	No	No
Protein aggregation	No	No	No	No
Water Distribution	No	No	No	No
Coumaric acid, 100 ppm, −10 °C				
PV	No	No	No	17% Reduction
TBARS	No	No	No	1.5% Reduction
Protein solubility	No	No	No	No
Protein aggregation	No	No	No	No
Water Distribution	No	No	No	No
Caffeic acid, 100 ppm, −18 °C				
PV	No	Yes, 37% reduction	Yes, 34% reduction	36% Reduction
TBARS	No	Yes, 18% reduction	Yes, 25% reduction	22% Reduction
Protein solubility	No	No	No	No
Protein aggregation	No	No	No	No
Water distribution	No	No	No	No
Coumaric acid, 100 ppm, −18 °C				
PV	No	No	No	−8%
TBARS	No	No	No	−2%
Protein solubility	No	No	No	No
Protein aggregation	No	No	No	No
Water distribution	No	No	No	No

namically feasible considering the low reduction potential of $AscH^-$ (E = 0.28 V), which enables to repair oxidising free radicals with greater reduction potential, including CaA (E = 0.54 V). Electron spin resonance (ESR) spectroscopy experiments have confirmed a higher capacity of CaA to regenerate α-TOH via reduction of the α-tocopheroxyl radical compared to other cinnamic acid derivatives (o-coumaric, ferulic and chlorogenic acids (Iglesias et al., 2009).

Redox cycles can be limited by an effective contact between oxidants and reductants in order to establish redox interactions. CaA is localised at the phospholipid-water interface of biological membranes, α-TOH is mostly located in the outer monolayer with the chromanol ring oriented to the aqueous phase, and the hydrophilic $AscH^-$ is localised in the water phase. Therefore, this localisation of CaA facilitates both interactions with α-TOH and $AscH^-$ in muscle tissues. The entire process results in a stronger antioxidant protection against lipid oxidation by favouring, as a final point, the protection of α-TOH, which is suggested as the last defence of fish muscle against lipid oxidation.

In the washed cod mince system, aqueous components such as ascorbic acid were lost during the washing steps. Therefore, the redox cycle is denied and this could explain the minor CaA effectiveness found in this system at frozen temperatures compared with minced unwashed fish muscle.

The antioxidant ability of CaA in horse mackerel mince was highly dependent on the antioxidant/lipid substrate ratio in horse mackerel mince. CaA-concentrations below 100 ppm seemed to be sufficient to significantly inhibit the progress of lipid oxidation when the lipid content was $\leqslant$ 2%. When the lipid concentration was $\geqslant$ 2%, the antioxidant effectiveness of CaA showed a significant

Table 5
Effect of CaA (75, 150 or 300 mg/kg) on lipid oxidation on fitness bars enriched with 5% fish oil stored up to 10 weeks. The data are related to controls with no addition of phenolic acids. Adapted from Horn et al. (2009).

Measure of lipid oxidation	Prolongs lag phase	Effect on max level	Effect on rate	% Inhibition after 10 weeks of storage
PV	No	Yes, increase max level	Yes, increase rate from start of experiment	75 ppm: −21% 150 ppm: −33% 300 ppm: −13%
Volatiles exemplified by 1-penten-3-ol	No	Yes increase max level	Yes, rate increased from day 0	75 ppm: −190% 150 ppm: −640% 300 ppm: −380% All after 10 weeks of storage
Rancid odour and taste	No	Yes, increase max level	Yes, rate increase from day 0	75 ppm: Rancid odour: −56% Rancid taste: −37% 300 ppm: Rancid odour: −29% Rancid taste: −23% 150 ppm not evaluated All after 10 weeks of storage
α-Tocopherol				Yes, alpha and gamma (slightly) tocopherol consumption increased The consumptions from week 0 to week 10 were (μg/g): Control: 56 (α), 0 (γ) 75 ppm: 59 (α), 11 (γ) 150 ppm: 72 (α), 5 (γ) 300 ppm: 75 (α), 7 (γ)

reduction which confirms that a phenolic compound to lipid ratio of 1:200 is needed for optimal antioxidant effect (Medina et al., 2009).

Antioxidant effectiveness of CaA was also dependent on the physical state of the horse mackerel mince, chilled or frozen (Medina et al., 2009). In general, the effectiveness of CaA was lower in frozen than chilled fish. There were no large differences in the relative inhibition provided by CaA between −10 °C and −18 °C storages. Freezing temperatures can reduce the diffusion of the compounds to oxidation sensitive sites such as membranes (Frankel, 1998). At low temperatures, diffusion is lower, and may be slowed further due to a higher resistance of the tissues to mass transfer (Ramesh & Duda, 2001). In frozen systems the non-frozen fraction may be highly viscous, and diffusion may become a limiting factor in some reactions. In addition, protein aggregation and denaturation occurring during frozen storage could modify the surface of membranes and then reduce the interaction of CaA with membranes. The capacity of antioxidants to induce changes in membranes and provoke dehydration effects has been related to decrease of the accessibility of prooxidant molecules into the hydrocarbon chain of PUFAs, hindering lipid radical propagation inside the chains (Maestre, Micol, Funes, & Medina, 2010).

Results obtained by Larsson and Undeland (2010) showed that CaA was an excellent inhibitor of Hb-mediated oxidation in washed cod mince, however, it did not prevent Fe-mediated oxidation in this system (Jacobsen et al., 2008). These results were in agreement with the above mentioned studies on liposomes (Kristinová et al., 2009) in which a CaA mediated-reduction of Fe^{3+} to the more active Fe^{2+} has been cited.

CaA has been demonstrated to inhibit protein carbonyl formation and loss of salt solubility in chilled washed cod mince (Larsson & Undeland, 2010). However, during frozen storage, salt solubility losses were not prevented in this system. In frozen minced horse mackerel, no support was found suggesting that CaA provoked a reduction on loss of salt solubility, protein aggregation or water holding capacity (Medina et al., 2009). Protein aggregation was not accompanied by gross protein denaturation. These data showed no evidence of a direct relationship between prevention of lipid oxidation in frozen fatty fish due to the addition of a highly polar antioxidant as CaA and reduced texture deterioration.

4.2.5. Activity of caffeic acid in a fish oil fortified fitness bar

Table 5 shows the results obtained from the application of CaA to fish oil fortified fitness bar (Horn, Nielsen, & Jacobsen, 2009). CaA showed a high prooxidant activity demonstrated by the promotion of peroxides, volatiles, as well as rancid odour and taste. It also increased the consumption of tocopherol. The prooxidative effect of CaA was suggested to be due to its ability to reduce Fe^{3+} to Fe^{2+}, but the hydrophilic nature of CaA and its possible location outside the oil droplet may also have affected its activity. Thus, it is expected that after emulsification of the oil–water emulsion delivery system, CaA was mainly partitioning into the water phase, although it to some extent may have adsorbed to the oil–water interface. When the emulsion is poured into the energy bar dough, CaA is expected to be located outside the oil droplets, and thus be in close proximity to the transition metal ions present in the other ingredients. These metal ions will subsequently catalyse oxidation. The content of iron was found to be relatively high in the energy bars, and this could also support the hypothesis, that a prooxidative effect of CaA is related to the reduction of transition metal ions. Moreover, if CaA becomes oxidised itself, other phenolic compounds present in ingredients like rolled oats and raisins may use their antioxidative capacity to regenerate CaA instead of lipid radicals. This will reduce the antioxidative effect of these other phenolic acids. However, the actual availability of iron in the energy bars as well as the actual location of iron and CaA in the energy bars deserve further investigation.

5. Conclusions

CaA has been demonstrated to act as an effective antioxidant in fish minces stored at cold temperatures. In the fish muscle based studies reviewed, the antioxidant effectiveness of CaA was higher than that showed by other hydroxycinnamic acids such as o-coumaric acid, ferulic acid and chlorogenic acid. Interestingly,

the antioxidant activity of CaA was very different in bulk fish oil, fish oil-in-water emulsions and cod roe phospholipid liposomes in which the CaA was scarcely active against lipid oxidation. Thus, the studies reviewed here show that the capacity of CaA to protect marine lipids against oxidation is highly dependent on the physical state of the marine lipids, and also the intrinsic medium in which they are found. CaA can significantly prevent oxidation fish muscle foods and is clearly a strong inhibitor against Hb-mediated oxidation. However, its activity in food emulsions and liposomes is highly dependent on the pH, the emulsifier used and the prooxidants present; together with low molecular weight Fe, CaA is rather a pro- than antioxidant and thus should be used with caution.

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