Revilija Mozuraityte

Oxidation of marine phospholipids in liposomes

Thesis for the degree of doktor ingeniør

Trondheim, June 2007

Norwegian University of
Science and Technology
Faculty of Natural Science and Technology
Department of Biotechnology

NTNU
Innovation and Creativity

NTNU
Norwegian University of Science and Technology

Thesis for the degree of doktor ingeniør

Faculty of Natural Science and Technology
Department of Biotechnology

ISBN 978-82-471-3134-3 (printed ver.)
ISBN 978-82-471-3148-0 (electronic ver.)
ISSN 1503-8181

Theses at NTNU, 2007:143

Printed by Tapir Uttrykk

Acknowledgements

I would like to express my appreciation and gratitude to many people for help and support.

First of all, I would like to thank to my supervisors, Professor Turid Rustad (Department of Biotechnology, NTNU) and Senior Researcher Ivar Storrø (Sintef Fisheries and Aquaculture) for their support, encouragement and guidance.

I send ‘thank You!’ to people from the Lipidtext group in the Integrated Research Project SEAFOODplus for interesting discussions in the research area of lipid oxidation.

It was a pleasure to be introduced to the world of Mass spectrometry analyses and for this opportunity I say ‘Thank You’ to Kolbjørn Zahlsen (Head of analytical research and development, Dept. of Clinical Pharmacology, St Olav’s University Hospital).

During the last year of my studies I have also been working part time on the research projects at Sintef, for this great opportunity I want to thank Dr. Marit Aursand.

I wish to thank to all my friends. Special gratitude I express to Modestas, for love and readiness to help.

Finally, but most importantly, I would like to thank to my parents, for love, support and care, and for always believing in me.

Summary

Marine phospholipids contain a high amount of n-3 polyunsaturated fatty acids (PUFAs), which have documented beneficial effect on human health. Due to this, marine phospholipids have a high potential for use in products for human consumption. However, due to the high amount of n-3 PUFAs, marine phospholipids are very susceptible to lipid oxidation, which cause loss of sensory and nutritional value in foods, some oxidation compounds are even toxic. A successful incorporation of marine phospholipids in processed foods would most probably be in the form of lipid dispersions, where some common constituents such as iron would be present. Iron, which is a very important mineral from a nutritional point of view, is also a very strong prooxidant. Studies of oxidation in oil/water systems catalysed by iron would provide valuable information on oxidation kinetics.

This thesis summarises the work done on oxidation of marine phospholipids in liposomes. Measurement of dissolved oxygen uptake was chosen as the main method to study lipid oxidation. This fast and simple method enabled screening of the influence of different conditions on oxidation.

The mechanism for iron catalyzed oxidation in liposomes is discussed. When Fe^{2+} was added to liposomes, an initial drop in dissolved oxygen followed by a slower linear oxygen uptake, was observed. Addition of Fe^{3+} induced only the linear oxygen uptake. The initial fast drop in dissolved oxygen was due to breakdown of pre-existing lipid peroxides by Fe^{2+}. In this reaction alkoxy radicals were formed and Fe^{2+} was oxidised to Fe^{3+}. Fe^{3+} formed was further reduced by peroxides to Fe^{2+} at a slow rate (compared to Fe^{2+} oxidation rate). When equilibrium between Fe^{2+} and Fe^{3+} was achieved, the linear oxygen uptake was observed and Fe^{3+} became the rate limiting factor in the circulation between Fe^{3+} and Fe^{2+}. Both alkoxy and peroxy radicals are presumably formed by breakdown of peroxides by Fe^{2+} and Fe^{3+}. These radicals react with fatty acids giving a lipid radical reacting with oxygen.

The rate of slower linear oxygen consumption followed Arrhenius kinetics and the variation in activation energy found (60 – 87 kJ/moles*K). The rate of slower linear oxygen uptake in liposomes was proportional to the concentration of iron and the lipid concentration in the assay mixture. The oxygen consumption rate was dependent on pH with a maximum observed between pH 4 and 5. The pH effect was explained by the iron availability and Zeta potential changes at different pH. Different salts had different influence on the linear oxygen uptake in liposomes. Cations (Na^{+},

K^+, Ca^+, Mg^+) did not influence the rate of oxidation in the tested range (Ionic strength (I) 0 - 0.14 M). Among the tested anions: sulphates and nitrates did not change oxygen uptake rate significantly, but chlorides (KCl, NaCl, $CaCl_2$) reduced the oxidation rate down to approximately 45 % and dihydrogen phosphate down to 14 %, when I=0.14M. The effect of Cl^- and $H_2PO_4^-$ was additive. When the liposomes contained different concentrations of chlorides, a linear relationship between oxygen uptake rate and Zeta potential was observed. When phosphate was added, the oxygen uptake rate was not related to the changes in Zeta potential.

The influence of pH, temperature, concentration of NaCl, phospholipids and Fe^{2+} on slower linear rate were described by mathematical equations. The modelled data based on the described equations fitted within 10% standard deviation with observed values.

CONTENTS

List of papers:

Paper I Mozuraityte R., Rustad T., Storrø I. (2006) Pro-oxidant activity of Fe^{2+} in oxidation of cod phospholipids in liposomes. *Eur. J. Lipid Sci. Technol.* 108, 218-226

Paper II Mozuraityte R., Rustad T., Storrø I. (2007) The role of iron in peroxidation of PUFAs in liposomes. *Lipids* (submitted)

Paper III Mozuraityte R., Rustad T., Storrø I. (2006): Oxidation of cod phospholipids in liposomes: Effects of salts, pH and zeta potential. *Eur. J. Lipid Sci. Technol.* 108, 944-950

Paper IV Høy M., Mozuraityte R., Segtnan V., Storrø I., Mevik B. H., Rustad T., Næs T. (2006): The use of experimental design methodology for optimisation of a lipid oxidation rate assay. *Chemometr. Intell. Lab. System.* (submitted)

Abbreviations

E_a - Arrhenius activation energy
EPA- eicosapentaenoic acid
ESR - electron spin resonance
DHA- docosahexaenoic acid
DTAB - dodecyltrimethylammonium bromide
I – initiator
$L^{\bullet}$- lipid radical
$LO^{\bullet}$- alkoxy radical
$LOO^{\bullet}$- peroxy radical
LC-MS - liquid chromatography mass spectrometry
ESI-TOF-MS - electron spray injection time-of-flight mass spectrometry
LH- unsaturated lipid
LOOH - lipid peroxide
MES - 2-morpholinoethanesulfonic acid
PC - phosphatidylcholine
PE - phosphatidylethanolamine
PUFA - polyunsaturated fatty acid
PV – peroxide value
TBARS – thiobarbituric reactive substances
TLC – thin layer chromatography
NMR- nucleo magnetic resonance

1. BACKGROUND

1.1. Marine phospholipids

Marine lipids contain a high amount of polyunsaturated n-3 fatty acids, which have a documented beneficial effect on human health. The health benefits of n-3 fatty acids include reduced inflammatory (Boissonneault, 2000) and coronary heart (Kris-Etherton et al., 2002) diseases, reduced susceptibility to mental illness (Peet and Stokes, 2005) and improved brain and eye function in infants (Innis, 1991). These fatty acids are also used in antipsychotic treatment (Peet and Stokes, 2005). Due to the beneficial health effects of the n-3 fatty acids, there is an increasing interest in the food industry to incorporate fats rich in n-3 fatty acids in food products.

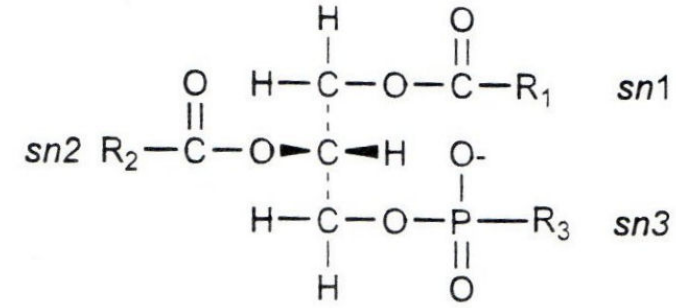
Marine phospholipids also contain high amounts of polyunsaturated n-3 fatty acids, mainly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3). The interest in marine phospholipids as carriers of n-3 fatty acids increases as n-3 fatty acids from phospholipids were observed to be more easily accessible for catabolic processes than n-3 fatty acids from triglycerides (Løvaas, 2006). The studies of lipid metabolism in infants also showed higher DHA absorption from phospholipids (n-3 enriched eggs) than from breast milk (Makrides et al., 2002). Therefore marine phospholipids can become an important marine ingredient in functional foods and have a high potential for use in products for human consumption, for use as delivery systems for drugs and in pharmacology (Løvaas, 2006).

Phospholipids, bilayer structured, are the main constituents of biological membranes, which serve as biological boundaries, responsible for metabolic regulation (Berdanier, 2000). Phospholipids in fish are found at relatively constant levels ~0.5-1% (w/w) so that in lean fish muscle the phospholipids make up most of the muscle fat. Antarctic krill (*Euphausia superba)* and fish roe are examples of raw material rich in marine phospholipids. The lipid content in krill may vary between 12.5 and 0.2% (w/w wet mass) (Pond et al. 1995) and the total polar lipid concentration may be as high as up to 70% of the total lipids (Phleger et al. 2002). The total lipid content in roes can make up to 19% of the wet weight depending on the source (Bledsoe et al 2003). The total lipids from cod roe and milt contain more than 70% of phospholipids (Tocher and Sargent, 1984, Bledsoe et al., 2003) and are good sources for marine phospholipids.

Phospholipids are often added to food because of their emulsification properties, such as for example soybean lecithin and egg yolk, which are common natural emulsifiers. Marine phospholipids could also be used as emulsifiers in food products. Due to the emulsification properties of marine phospholipids they can be incorporated in dry food matrices while for incorporation of marine triacylglyserols, emulsifiers have to be added. However, as marine phospholipids contain high amounts of polyunsaturated fatty acids (PUFAs) they are also susceptible to lipid oxidation contributing to the potential damage both in biological and in food systems. Lipid oxidation breaks down membranes in food systems, which leads to increased membrane permeability, cross-linking, polymerization of proteins etc. Another consequence of oxidation is generation of off-flavours, colour deterioration and loss of antioxidants in food (Erickson, 1998). In addition, some oxidation products have been observed to be toxic and have a negative effect on human health (McClement and Decker, 2000, Pearson et al., 1983). Therefore lipid oxidation limits the utilization of marine phospholipids both in processed foods, and as nutritional supplements. There is therefore a need to gain more knowledge on oxidation of phospholipids to be able to choose better storage and processing conditions and to find improved or optimal recipes for foods enriched with marine phospholipids.

1.2. Phospholipid chemistry

Triacylglycerols and phospholipids contain fatty acids that are the oxidizable substrate. Glycerophospholipids have two long – chain fatty acid residues, esterified with a glycerol molecule that also carries a phosphate group (Figure 1). The phosphate is almost always linked to the sn-3 position of the glycerol molecule. The phosphate group is in turn esterified with one of a number of organic bases, amino acids, or alcohols, which add to the hydrophilic character of the molecules. The hydrophilic group in many phospholipids includes both phosphate groups (negatively charged at the pH values normally found in food) and quaternary nitrogen atoms (positively charged) and is therefore classified as zwitterionic. Some common phosphoglyceride structures and nomenclatures are given in Figure 1.



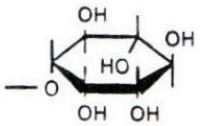
R_3 =	phospholipid class/nomenclature
-OH	phosphatidic acid (PA)
$-O-CH_2-CH(NH_2)-COOH$	(3-*sn*-phosphatidyl)-L- serine (PS) 1.2-diacyl-*sn*-glycero(3)phospho-L-serine
$-O-CH_2-CH_2-NH_2$	(3-*sn*-phosphatidyl)ethanolamine (PE) 1.2-diacyl-*sn*-glycero(3)phosphoethanolamine
$-O-CH_2-CH_2-N^+(CH_3)_3$	(3-*sn*-phosphatidyl)choline (PC) 1.2-diacyl-*sn*-glycero(3)phosphocholine
	(3-*sn*-phosphatidyl)-L-*myo*inositol (PI) 1.2-diacyl-*sn*-glycero(3)phospho-L-*myo*-inositol

Figure 1. Nomenclature of glycerophospholipids (from O'Keefe, 1998).

1.3. Liposomes

Due to their amphiphilic structure, phospholipids form bilayer vesicles, called liposomes (Figure 2). The name liposomes derives from two Greeks words (lipo- fat, and soma-structure) meaning hollow vesicles surrounded by a fatty envelope with an aqueous core (Arnaud, 1995).

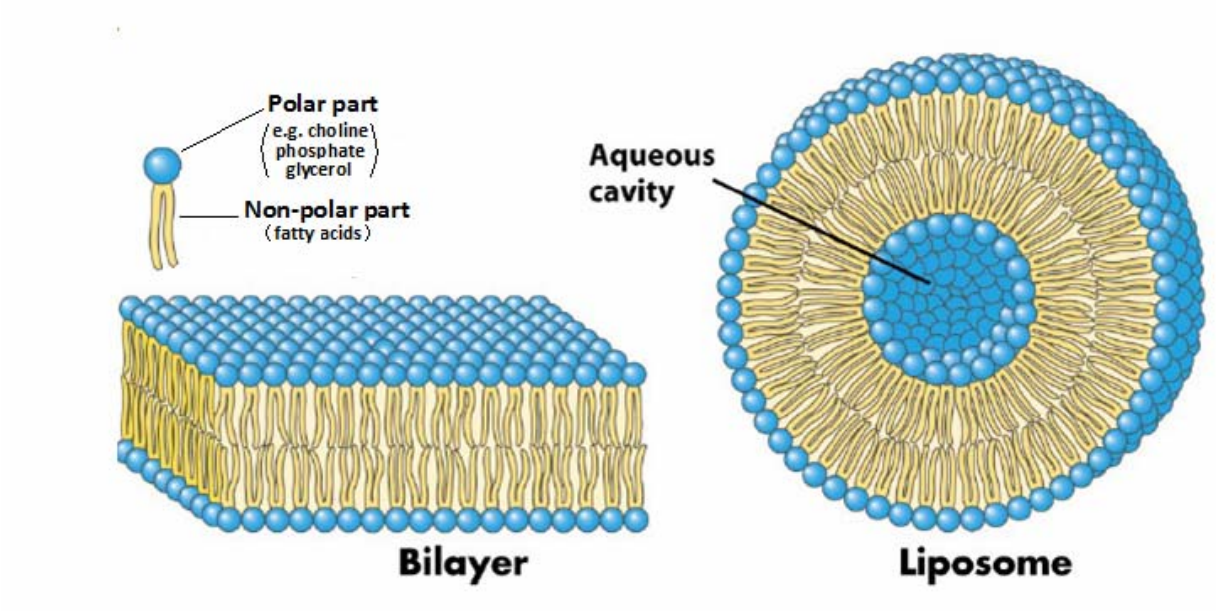


Figure 2. Bilayer structure of liposomes

1.3.1. The use of liposomes

The interest in using liposomes with entrapped food ingredients (enzymes, flavours, minerals and vitamins) for human and animal nutrition are likely to grow (Arnaud, 1995, Sato and Sunamoto, 1992).

- Different liposome formulations have been assayed as bioencapsulation products to enrich *Artemia* nauplii with nutrients for feeding fish larvae (Monroig et al., 2003).
- Cansell et al. (2003) pointed out that marine phospholipids might constitute an attractive material for development of liposomes as an oral PUFAs supplement due to the observed higher lipid bioavailability from liposomes compared to fish oil.
- Tocopherol as supplement should be taken with food. The absorption of fat soluble vitamins, including tocopherol, increases in the presence of surfactants (Bateman et al., 1984). Facilitated α-tocopherol uptake by liposomes (made from marine phospholipids) after oral delivery to rats compared to sardine oil ingestion was observed by Nacka et al. (2001). Due to this, liposomes were also envisaged as a potential form for α – tocopherol supplement.

- An iron source (non-heme) encapsulated in liposomes was observed to have a potential for reduction of iron – deficiency (Mehansho, 2006, Zimmermann, 2004).

However, due to the high susceptibility to oxidation for marine phospholipids in liposomes, there is a need for more knowledge about the oxidation kinetics in liposomes at different conditions to be able to utilize liposomes containing PUFAs as food supplements.

1.3.2. Liposomes – model system for studies of lipid oxidation

Studies of lipid oxidation in food systems are complicated due to the complex nature of most food systems with many components interacting and having an effect on the lipid oxidation (Frankel, 2005). Model systems such as bulk fish oils, emulsions, bilayer structures (microsomes, marine PL-liposomes) and fish minces have been used to simulate foods or biological samples in research on oxidation of marine lipids. The results obtained using model systems can be misleading because of oversimplified conditions. However, careful choice of model systems and storage conditions can help to identify the factors - that is which compounds and conditions - that have an effect on the oxidative instability of lipids, and indicate oxidative pathways in lipid oxidation (Decker and Hultin, 1992). By combining results from different model systems a more complete understanding of lipid oxidation in food can be obtained.

Successful incorporation of n-3 fatty acids into processed foods would most probably be in the form of lipid dispersions (Tong et al., 2000). Many studies have been done on oxidation in fish oil emulsions by the research group of McClement and Decker (Donnelly et al., 1998, Tong et al., 2000, Nuchi et al., 2001, Hu et al., 2003, Faraji et al. 2004, Djordjevic et al 2004, Kellerby et al., 2006), the research group at DIFRES (Jacobsen 1999, 2000a,b) and other research groups. However, because different emulsifiers can lead to differences in particle size distribution, and differences in physical and chemical characteristics of the interface (McClement, 1999) it can therefore be difficult to interpret the results of these studies.

Liposomes as a model system for lipid oxidation studies has the advantage that the same molecule serves both as oxidizable substrate (fatty acids) and emulsifier. Since there is no need for an emulsifier, the influence of the emulsifier on the oxidation is

avoided. Using liposomes in studies of lipid oxidation allows easy manipulation of lipid composition, pH, temperature, content of various agents such as salt, antioxidants, prooxidants etc. The influence of different factors on oxidation in emulsions and liposomes is discussed in section 1.4.3. Oxidation in liposomes could also provide relevant information on the mechanism that may influence the progress of the reaction in emulsions (Genot, 2003).

As liposomes have a high oxidative instability, they may be exploited for the examination of various types of oxidative stress and in the evaluation of antioxidant efficacy. Due to this liposomes can also serve as a model system for screening of antioxidant capacity (Løvaas, 2006).

Liposomes made from phospholipids mimic the structure of the cell membrane, where some proteins and antioxidants can be incorporated. The studies of the influence of tocopherol, carotenoids etc. on lipid oxidation in liposomes, can provide knowledge on their influence on oxidation in cell membranes (Azuma et al., 1999). Liposomes with incorporated proteins have also been used as a model system to study lipid-protein interaction in biological membranes (Chatterjee and Agarwal, 1988).

The knowledge obtained from studies of lipid oxidation in liposomes can also be useful for employing liposomes as a supplement of some vitamins, n- fatty acids, minerals etc.

1.3.3. Electrostatic characterization of liposomes

The polar head groups of phospholipid can be ionised and carry a charge. pK values of ionized phospholipids are given in Table 1. The pK values quaternary ammonium of choline residue carries a positive charge almost over whole pH scale and PC is a zwitterion over the pH 3 and PE at pH 2 - 8 (Tocanne and Teissie, 1990).

Table 1. pK values of ionized groups of phospholipids (Gibrat and Gignon, 1982),

	Dissociable group			
	$-PO_4^-$	$-PO_4^{2-}$	$-NH_3^+$	$-N^+(CH_3)_3$
pK	1.2	8.0	9.0	12.0

The polar region, where the polar head groups are located, constitutes an interface between the hydrophobic core of the membrane and the aqueous phase. In this region ionization of phospholipids and absorption of ions to the lipid head group generates an electrostatic surface potential, which is linked to the chemical structure of the polar head groups and the concentration of cations and anions in the aqueous phase (Tocanne and Teissie, 1990), Figure 3. Electrostatic force causes a firm attraction of oppositely charged particles (counter-ions) around the surface and this layer of counter ions is known as the *Stern layer*. Additional counter ions are also attracted by the colloid particle (liposome) but these are repelled by the Stern layer as well as by other counter-ions trying to approach the particle. This is called the *diffuse layer*, where the high concentration of counter-ions near the surface gradually decreases with distance until it reaches equilibrium. As the particle moves through solution, either due to gravity or to an applied voltage, some ions move with particle. The boundary, beyond which the ions do not move with the particle is called the *shear plane* and this is between the Stern layer and the diffuse layer. A charged particle will move with fixed velocity in a voltage field. The particle mobility is related to the dielectric constant and viscosity of the liquid and to the electrical potential at the boundary between the moving particle and the liquid. The Zeta potential is the potential that exists at the shear plane of the particle. The Zeta potential of the liposomes has been measured to determine the charge density of their surface, the binding affinity of various ions and it can also provide information on the structure of the lipid head group (Makino et al. 1991).

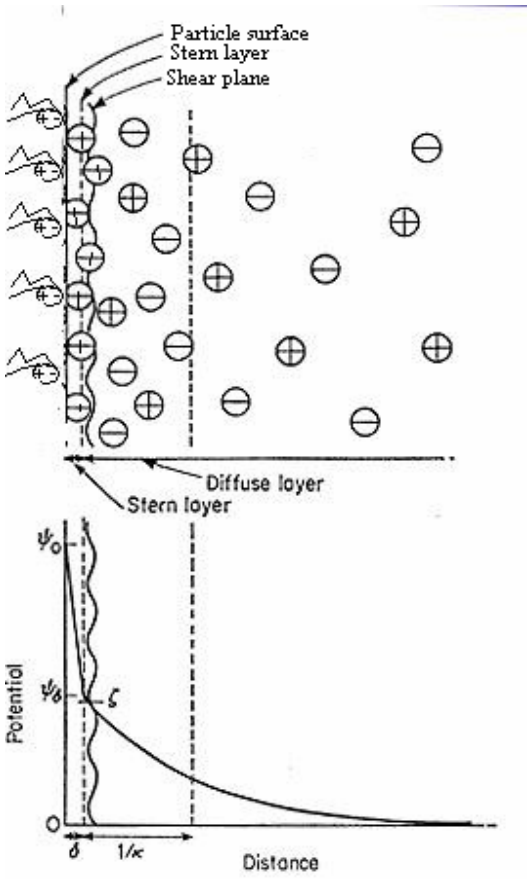


Figure 3. Schematic representation of the distribution of ions around the liposome surface.

1.4. Lipid oxidation

Depending upon the type of oxygen involved, autoxidation and photosensitized oxidation are responsible for the lipid oxidation during processing and storage of food products (Choe and Min, 2006). Two types of oxygen can react with lipids: triplet oxygen and the singlet oxygen. Atmospheric triplet oxygen reacts with lipid radicals and cause autoxidation, which is a free radical chain reaction. Photosensitised reactions, which are initiated by the presence of light and sensitizers, produce singlet oxygen from atmospheric triplet oxygen. Singlet oxygen can then react with fatty acids and lipid hydroperoxide is formed. Photooxidation is reviewed by Min (1998), Frankel (2005) and Choe and Min (2006).

When lipids oxidise, they react with oxygen producing peroxides as primary oxidation products. The peroxides can be broken down to secondary oxidation products such as ketones, aldehydes and alcohols. Homolytic β- cleavage of hydroperoxides leads to formation of volatile oxidation in the products, which cause off flavour in the products.

Contact between lipid oxidation products and proteins, vitamins etc, can result in various types of damage: protein denaturation, polymerisation, loss of vitamins, changes in texture, color etc. Oxidation of essential fatty and amino acids results in loss of nutritional value of food products (Frankel, 2005).

In food systems, lipids are usually dispersed in heterogeneous matrices such as for example muscle tissue, in emulsions and in powders (McClements and Decker, 2000). The organization of lipid molecules within the system and interaction with other molecules (prooxidants, antioxidants) has an influence on the susceptibility to oxidation. The influence of different factors on oxidation will be discussed later. When studying lipid oxidation, antioxidants are often the main focus, while the effects of pro-oxidants are often overlooked. There are many factors that can influence the oxidation of lipids. Among these are light, temperature, enzymes, metals, metalloproteins and microorganisms (Vercellotti et al., 1992).

1.4.1. Iron

Iron catalyses oxidative changes in lipids, but it is also a common constituent in food. It can be added in food as part of the water or with some food ingredients such as impurities in salts. Muscle foods also contain a high amount of iron. Most of the iron in animal tissue is found in hemoproteins, however, a number of substances also contain nonheme iron (ferritin, transferrin etc).

Iron is essential for life because it is required for oxygen transport, respiration and activity of some enzymes (Decker and Hultin, 1992). Iron deficiency is one of the most widespread nutritional deficiencies. Iron fortification of food has been recommended as one of the preferred approaches for preventing or reducing iron deficiency (Mehansho, 2006). However, food fortification with iron could cause oxidation mediated rancidity of fats and other related negative changes in foods.

As most of the food systems and biological membranes often contain polyunsaturated fatty acids and are surrounded by liquid rich in oxygen and metal ions (Chatterjee and Agarwal, 1988), it is also very important to gain more knowledge about the effects of different factors on iron induced lipid peroxidation. Studies of iron induced lipid oxidation can provide knowledge, which can help to choose better storage and processing conditions for food products, especially for those enriched with iron. This will make it possible to make high quality, stable products, including iron fortified products.

1.4.2. Free radical oxidation

Free radical oxidation can be described in terms of initiation, propagation and termination. These processes consist of a complex series of sequential and overlapping reactions (Frankel, 2005).

1.4.2.1. Initiation

Lipid peroxidation is the process in which molecular oxygen is incorporated into unsaturated lipids (LH) to form lipid hydroperoxides (LOOH). In terms of the onset of rancidity in food lipids the initiation is the most important step. The direct oxidation of unsaturated fatty acids (LH) by triplet oxygen is spin forbidden because the lipid ground state of single multiplicity has an opposite spin direction from that of oxygen of triplet multiplicity.

This spin barrier between lipids and oxygen can be readily overcome in the presence of initiators (I) (reactive oxygen species or transition metal) that overcome the dissociation energy of the allylic bond and thus cause abstraction of hydrogen atom forming a carbon-centered lipid radical ($L^{\bullet}$) (1) that can react with molecular oxygen to form a conjugated diene hydroxyl radical species (Minotti and Aust, 1999, Frankel, 2005).

$$LH \xrightarrow{I} L^{\bullet} + IH \qquad (1)$$

The greater the number of double bonds in a fatty acid side chain, the easier is the removal of a hydrogen atom, which is why PUFAs are particularly susceptible to peroxidation (Frankel, 2005). Once a lipid radical is formed, the chain reaction can begin to propagate.

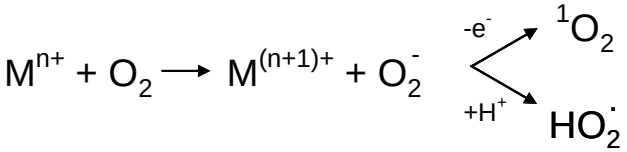
1.4.2.2. Iron catalysed lipid oxidation

Food lipids generally contain trace amounts of heavy metals probably arising from the presence of metal activated enzymes or their decomposition products (Ingold, 1962). Heavy metals, particularly those with two or more valency states, increase the rate of lipid oxidation. The mechanism of iron-catalysed lipid peroxidation is proposed to depend on the presence or absence of preformed lipid hydroperoxide (LOOH) and is divided into LOOH-independent (or iron dependent) and LOOH-dependent initiation (Girotti, 1985).

LOOH - independent oxidation occurs in lipid without peroxides. The mechanism of LOOH – independent initiation is still not clear.

Transition metals are capable of directly breaking down unsaturated lipids (LH) into alkyl radicals ($L^{\cdot}$), but this reaction occurs slowly and is therefore not believed to be important in promoting lipid oxidation (Reische et al., 1998).

One pathway of LOOH-independent initiation can be through oxygen activation in the presence of transition metals, when initiation of the oxidation occurs either by formation of peroxy radical ($HO_2^{\cdot}$) or singlet oxygen ($^1O_2$) (Nawar, 1996):



However, peroxy radical was observed not to play a major role as initiator of peroxidation in liposome systems (Aruoma et al. 1989).

Minotti and Aust (1987) proposed that Fe^{2+} will not promote lipid peroxidation in lipid peroxide free lipids and that this requires both Fe^{2+} and Fe^{3+}. However, Tadolini and Hakin (1996) argued against the participation of Fe^{3+} in the initiation of peroxide free lipid peroxidation.

The formation of an Fe^{2+}-O_2-Fe^{3+} complex (Minotti and Aust, 1987, Yin et al., 1992) and iron-oxygen complexes such as ferryl ion and perferryl ion (Goddard and Sweeney, 1987) which initiate lipid oxidation, have been suggested. However, the proposed iron complexes have not been isolated (Ohyashiki et al., 2002), and the findings (Aruoma et al., 1989) indicated that a specific Fe^{2+}- Fe^{3+} complex could not be required for oxidation initiation.

Radicals as $OH^{\bullet}$, $O_2^{\bullet -}$, which can be generated during the Fenton reaction or by autoxidation of ascorbic acid/Fe^{2+}, were also observed not to be involved in the process of lipid peroxidation (Ohyashiki et al., 2002, Fukuzawa et al., 1993, Yin et al., 1992).

Several studies (Fukuzawa et al., 1988, Fukuzawa et al., 1993, Tadolini et al., 1997) have shown that when peroxides were removed from the lipids, lipid peroxidation was not induced by iron. However, even with a low amount of peroxides, the peroxide dependent oxidation reactions may become dominant. As almost all lipids contain at least traces of peroxides, decomposition of peroxides by iron can be the most important cause of oxidation in many foods (Decker and McClement, 2001).

LOOH dependent initiation has been proposed to occur through two pathways:

a) LOOH breakdown by Fe^{3+} and subsequent hydrogen abstraction by $LOO^{\cdot}$ (reaction 1 and 2);

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + H^{+} + Fe^{2+} \quad (1)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (2)$$

or

b) Fe^{2+} accelerates lipid oxidation by breaking down lipid peroxides to free radicals (reaction 3 and 4)

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^{-} + Fe^{3+} \quad (3)$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \quad (4)$$

A possible circulation between Fe^{2+} and Fe^{3+} through peroxide decomposition reactions 1 and 3 was proposed by Pokorny (1987) to be an important source of free radicals, but this theory does not seem to have attracted much attention.

Fe^{2+} is reported to decompose peroxides at a higher rate than Fe^{3+} ions (Frankel, 2005). This could be attributed to a lower activation energy for the cleavage of O-O bond (184 kJ/mol) than that for the O-H bond (377 kJ/mol) (Min, 1998).

A site specific initiation mechanism in oil/water systems was proposed in several studies (Garner-Suillerot et al, 1984, Fukuzawa et al.1993), in which the binding of iron to the membrane surface is a prerequisite for its reaction with peroxides. Alkoxy ($LO^{\cdot}$) and peroxy ($LOO^{\cdot}$) radicals formed as a result of peroxide decomposition by iron, can then penetrate the hydrophobic region and trigger the initiation reactions by abstracting further H atoms from fatty acid LH, with alkoxy radicals being more reactive than peroxy radicals (Buettner, 1993).

Tang et al. (2000) also proposed that reaction between Fe^{2+} and lipid peroxide was responsible for the initiation of lipid peroxidation, but lipid peroxidation could only start when the concentration of Fe^{2+} was reduced to a critical value. Before the critical Fe^{2+} concentration was reached, a latent period was observed. During this period, the concentration of Fe^{2+} was reduced to a level where the peroxy and alkoxy radicals could no longer be converted to non-radical species by their reaction with the remaining Fe^{2+} (5, 6) and their attack on the fatty acid chain became dominant (2, 4).

$Fe^{2+} + LOO^{\bullet} + H^{+} \rightarrow LOOH + Fe^{3+}$ (5)

$Fe^{2+} + LO^{\bullet} + H^{+} \rightarrow LOH + Fe^{3+}$ (6)

1.4.2.3. Propagation

Once a free radical $L^{\cdot}$ is produced, it can react with diradical triplet oxygen to form peroxy radical (Min, 1998).

$L^{\cdot} + O_2 \rightarrow LOO^{\cdot}$ (7)

The interaction of $L^{\cdot}$ with O_2 has a very low activation energy (Min, 1998) and the reaction rate is very fast (Ivanov, 1985). The peroxy radical formed can further subtract hydrogen from a fatty acid generating $L^{\cdot}$ (2) which can react with oxygen and produce another $LOO^{\cdot}$. Hence a single initiation event can result in conversion of hundreds of fatty acid side chains into lipid hydroperoxides, accompanied by oxygen consumption (Gutteridge and Halliwell, 1990). The decomposition of LOOH also results in the accumulation of short-chain lipid peroxidation end products, mostly aldehydes (Minotti and Aust, 1989).

1.4.2.4. Termination

At the last stage of autoxidation the peroxyl radicals accumulate and tend to interact with each other forming non-radical products. When oxygen is abundant, the reaction between alkoxy ($LO^{\cdot}$) or peroxy ($LOO^{\cdot}$) with a low activation energy (~4 kcal/mol) forms non radical oxidation products (Colakoglu, 2007). When the oxygen concentration is low, the reaction of $L^{\cdot}$ with $LOO^{\cdot}$, $LO^{\cdot}$ or $L^{\cdot}$ occurs and prevents the sequence of reaction from repeating (Colakoglu, 2007).

1.4.3. Major factors that influence iron induced lipid oxidation in oil in water systems

The understanding of the *factors that affect metal-hydroperoxide interactions* and therefore the iron induced lipid oxidation is still incomplete (Decker and McClements, 2001). Traditional chemistry holds that catalysis in a biphasic system will occur at the

phase interface or at the membrane surface. Peroxides are more polar than unoxidised fat and tend to accumulate near the surface. Thus reaction conditions, which increase binding of iron to the surface, will increase the prooxidative effect of iron (Schaich, 1992, Garnier-Suillerot et al., 1984).

1.4.3.1. The nature of the interface

Lipid oxidation in multiphase systems is greatly influenced by the nature of the interface (Frankel, 1998, McClements and Decker, 2000). The importance of *droplet charge* for lipid oxidation has been demonstrated in a number of studies (Mei et al., 1998a, b, Fukuzawa et al., 1988), where lipid oxidation was promoted when positively charged iron ions were electrostatically attracted to the surface of the negatively charged emulsion droplets (McClements and Decker, 2000). However, Mancuso et al. (2000) suggested that because of the high reactivity and solubility of iron it is not absolutely required to be electrostatically attracted to the emulsion surface as ferrous ions still reacted with peroxides in Tween (neutral) and dodecyltrimethylammonium bromide (DTAB, positive) stabilised emulsions (in which iron-emulsion droplet interaction are expected to be low).

In *in vitro* studies, membrane lipids have been shown to oxidise faster than emulsified triacylglycerols, because of the arrangement of phospholipids in membranes (Erikson, 1998). Membrane-liposome peroxidation was observed to depend on *the nature of the head group* of lipids (Fukuzawa et al., 1988). Inclusion of PE in liposomes was observed to accelerate the Fe^{2+} dependent peroxidation (Wang et al., 1994, Kawakatsu et al., 1984). The phosphoryl base of PE (which is partial anionic) was proposed to accelerate the decomposition of hydroperoxides by attracting the ferrous ions to the surface. The phosphoryl base of PC has a weaker attraction of iron because of its zwitterionic character over the entire pH range (Terao, 1989). Corliss and Dugan (1970) also observed that PE oxidised more rapidly than PC, but the activation energy for PC and PE from egg and soybean were not grossly different from each other, which suggest that the mode of oxidation was the same. However, addition of phospholipids to bulk oil even increased oxidative stability of the oil. The effect was attributed to chelation of metals by the phosphate group. Fatty acid chain oxidation produces carbonyls, which are able to react with the primary amino group of phospholipid producing compounds with antioxidative properties (Hidalgo et al., 2005).

The ability of iron to break down lipid hydroperoxides can also depend largely on the physical location of the peroxide relative to the interface of the emulsion or the liposome droplet (Garnier-Suillerot et al., 1984). Hydroperoxides, as surface-active molecules, have a tendency to migrate toward the surface of the droplet (Buettner, 1993). In this location, there is a greater opportunity for them to interact with and be decomposed by prooxidants.

A slower peroxide decomposition was observed in emulsions which contained emulsifiers with "larger" (higher numbers of polyoxyethylene groups) *hydrophilic head groups* (Silvestre et al., 2000). Longer *hydrophobic tail group* in emulsifiers can also decrease the ability of the free radicals, originating from peroxides decomposed by Fe^{2+}, to reach the polyunsaturated fatty acids and thereby increase the oxidative stability of emulsions (Chaiyasit et al., 2000). Thick two-layer interfacial membrane was also observed to provide improved oxidative stability of emulsions (Djordjevic et al., 2007). In liposomes, iron can bind to the phosphoric acid of phospholipids and this can lead to breakdown of lipid peroxides near this site (Scaich, 1992).

1.4.3.2. Droplet size

The chances of prooxidant attack on the interface of the oil droplet increases with increasing the surface area, therefore a smaller droplet size reduces the oxidative stability. Nevertheless, the number of lipid molecules per droplet decreases when droplet size is reduced. This could limit the propagation chain as radicals easier reacts with adjacent fatty acids in the same droplet than by transfer to another particle and oxidative stability could increase. Due to these conflicting views, some contradictions have been observed in studies on the influence on droplet size on the oxidation rate (Genot et al, 2003). Jacobsen et al. (2000a, b) observed a positive correlation between increased droplet size and oxidative stability of fish oil enriched mayonnaises in early stage of oxidation. Jacobsen et al. (2000b) concluded that droplet size is important only for oxidation initiation; the propagation of oxidation is independent of droplet size

1.4.3.3. The nature of fat phase

The fatty acids are of primary importance to the rate of development of rancidity, since they can be very susceptible (as polyunsaturated fatty acids) to attack by oxygen (Labuza, 1971, Frankel, 2005). It is generally accepted that the oxidative stability of

PUFA decreases with increasing degree of unsaturation (Frankel, 2005). However, in liposomes, the degree of unsaturation was observed not to be the main factor affecting the oxidation stability of PC (Araseki et al., 1999). Liposome oxidative stability was observed to be more influenced by the fatty acid conformation in bilayers than the surface area (Araseki et al., 1999). DHA in liposomes could form a tightly coiled packed confirmation and the higher reactivity of DHA could be compensated by difficulty of free radical and/or oxygen to attack this tighter confirmation (Araseki et al., 1999). Araseki et al. (1999) also predicted that the intramolecular hydrogen abstraction from unsaturated acyl group by a free radical of another acyl group in the same ester molecule occurs more rapidly than the intermolecular hydrogen abstraction, because acyl groups bound to the same ester molecule are closer to one another.

1.4.3.4. pH

The effect of pH on lipid oxidation is associated with the effect of pH on the availability of metals, the surface activity, and on ionic interaction (Nawar, 1996). The prooxidant activity of iron depends on chemical state, solubility, and physical location of iron (Mancuso et al., 2000). At low pH where iron solubility increases, the high degree of interaction between lipid hydroperoxides and iron in SDS – stabilized (negative charged) emulsions was observed to cause hydroperoxide decomposition almost as fast as their formation (Mei et al., 1998a). Oxidation rate in phospholipid emulsions decreased in the following order: pH 5.8>7>8 (Kawakatsu et al., 1984). However, in the studies of Mancuso et al. (1999), higher oxidation rate was observed at pH 7 than at pH 3. This was explained to be due to the low solubility of iron at pH 7 resulting in precipitation of metal on the lipid droplet surface, thereby bringing iron closer to the lipid compared to pH 3, where the iron solubility is dramatically higher.

1.4.3.5. Salts

Salts are commonly present in foods. NaCl is added to food products for a variety of purposes, including flavour and inhibition of microorganisms. However, little is still known about the effect of different ions on the kinetics of lipid oxidation and literature data are often contradictory. Several studies have analysed the influence of NaCl on the iron catalysed lipid oxidation in different emulsions. The antioxidant effect of NaCl was

explained by screening of electrostatic interaction between charged groups (McClement and Decker, 2000, Mei et al., 1998a), resulting in a reduced tendency of iron to come closer to the lipid particle surface or to reduced oxygen solubility (Hunter, 1993). However, no effect of NaCl was observed on oxidation of emulsions with positively charged emulsifiers in the studies of Mei et al. (1998a), indicating that neither sodium nor chloride was capable of altering the surface in a manner that increase iron –lipid interactions. A prooxidant effect of NaCl was also observed in some studies. This was proposed to be due to the ability of NaCl to increase the catalytic activity of iron (Osinchak et al., 1992). Hydroperoxides are more polar than the lipids from which they originate, and ionic compounds such as salts may change the hydroperoxide conformation and also affect the oxidation kinetics (Calligaris and Nicoli, 2006).

1.5. Methods to study lipid oxidation in aqueous colloidal system

A number of different analytical methods can be used for measuring oxidative deterioration. Methods can be classified in four groups based on what they measure (Dobarganes and Velasco, 2002):

- absorption of oxygen,
- loss of initial substrate,
- formation of hydroperoxides (primary oxidation products),
- formation of secondary oxidation products.

The most common methods to determine primary oxidation products are peroxide value (PV) and conjugated dienes. PV analyses can be performed using iodometric, ferric thiocyanate methods. The observed values are relative, because they have been observed to differ for different methods (Nielsen et al. 2003). The determination of peroxide value is useful for bulk oils that can be analysed directly. For other food systems such as emulsions, minced muscle, etc. the lipid is extracted with solvents. Solvent evaporation should be performed carefully without decomposition of peroxides (Frankel 2005). Conjugated diene hydroperoxides produced in PUFAs can be determined by their strong absorption at ~234nm. The sample is simply dissolved in methanol or isooctane and the absorbance measured. The measurements can be also performed directly on emulsion dissolved in methanol. The method is not suitable for oils with low amounts of PUFAs.

In many studies traditional peroxide and TBARS (thiobarbituric ractive substances) methods have been used for evaluation of oxidation induced by Fe^{2+} in aqueous colloidal systems.

TBARS and anisidine value evaluate secondary oxidation products. Several versions of the method to determine TBARS are employed. The basis of the methods is that one malonaldehyde molecule (oxidation product) reacts with two molecules of thiobarbituric acid forming pink colour complex, and the intensity is measured at 530nm. However, some proteins, antioxidants, sugars can also react with TBARS and influence the results (Frankel, 2005).

Peroxides can be further decomposed by scissions of hydroperoxide group giving an alkoxy radical. Homolytic β- cleavage of alkoxy radical gives low molecular weight, volatile compounds. These compounds are responsible for off-flavor development and have been investigated in a number of oxidation studies. Volatiles can be analysed by several types of headspace analyses (Frankel, 2005).

Schiff base compounds formed by the interaction of oxidation products with proteins, phospholipids and nucleic acids produce chromofores showing characteristic fluorescence spectra. Several fluorescence techniques have been used to investigate oxidation. This method is sensitive, but not specific as it measures complex mixtures resulting from interaction between different lipid oxidation compounds and proteins, peptides etc (Frankel, 2005).

Aldehydes, left on the triacylglycerol or phospholipid molecule after homolytic β-cleavage of the peroxide, are called core aldehydes. Core aldehydes does not directly contribute to off-flavor, but they can associate with cell membranes and affect their function and structure. The interest in studies of core aldehyde formation increases in medicine and in studies of food quality (Kuksis et al 2003). New techniques such as LC-MS, ES-TOF-MS, etc. have been used in studies of core aldehydes as well as in formation of other oxidation products.

High resolution nuclear magnetic resonance (NMR) can provide information on important changes in chemical composition during oxidation, however due to its low sensitivity it is difficult to use for early stage oxidation studies (Falch, 2006). As lipid oxidation proceeds with formation of free radicals, electron spin resonance (ESR) spectroscopy has been investigated for early lipid oxidation. A major limitation in the detection of free radicals by ESR is the requirement that radical concentration remains higher than 10^{-8} (Shahidi and Wanasundara, 1989). Several approaches have been used to

keep the steady concentration of radicals higher: spin traps, rapid freezing. The application of ESR to study lipid oxidation is quite new.

However, the methods mentioned above involve some preparation and/or development steps and are time consuming. The breakdown pathways of lipid oxidation products can vary dependent upon the physical and chemical conditions under which the oxidation take place. Changes in pathways make it difficult to rely on one or a few oxidation products to quantify lipid oxidation. Therefore is interesting to try to use the reaction substrate as an indicator for oxidation.

Decrease in concentration of fatty acids or oxygen could be possible to measure. The sensitivity in measuring oxygen concentration is in the range of 1-5μM, and close to the sensory threshold level for products formed from lipid oxidation (0.004-27 μM) (Kulås et al., 2003). However, when measuring fatty acids this sensitivity is hard to reach.

The measurement of oxygen absorption is available as one of the rapid methods for evaluating oxidation (Shimada et al., 1996). One of the earliest descriptions of the different stages of lipid peroxidation was given in the late 1820s by de Saussure, who used a simple mercury manometer to study the uptake of oxygen by a layer of walnut oil on water (Gutteridge & Halliwell, 1990). The polarographic method has the advantage to allow a direct, fast and continuous measurement of oxidation without any preparative steps such as is involved in calorometric or chromatographic methods (Genot et al., 1994). The oxygen absorption measurements have been used in a number of studies to evaluate oxidation kinetics (Yamamoto et al. 1984, Yoshida and Niki, 1992), effects of antioxidants (Genot et al., 1994, Yamamoto et al. 1988) or prooxidants in aqueous membrane model systems. Usually prooxidants such as metals are added to accelerate the oxidation which make the experimental time quite short. These accelerated methods are often criticised that because the results are obtained at higher temperatures and with excessive oxidation it is difficult to extrapolate the results to ambient storage conditions (Frankel, 2005). Unfortunately, time is usually a limited resource in research activities and some screening of an influence of different additives to an oxidation can provide knowledge on where more studies should be performed. Using fast methods may enable many different screening studies using different parameters (temperature, different food system preparation methods etc), combinations (pH, salts, antioxidants, prooxidants) and repetition. The availability of equipment also is an important factor in making a choice of the method.

1.6. Modelling of the data

Studying the influence of different factor on oxidation it is difficult to maintain the same experimental conditions such as pH, ionic strength. Due to this it is difficult to make conclusions which factors really affected the oxidation. Number of experiments should be investigated due to this. Mathematical modeling can reduce the number of experiments; thereby reducing time and expenses.

The first step in kinetically studying food oxidation is to determine the most important parameters that reflect the overall quality loss. Identifying and estimating the influence of these parameters a mathematical model can be developed. The main purpose to develop a robust and validated mathematical model is to expand its use at conditions that differ from experimental ones (Giannakourou and Taoukis, 2007). By comparing the calculated data with the observed ones, the suitability of the model can be established.

2. AIM OF THE WORK

This work was performed within the Integrated Research Project SEAFOODplus, contract No FOOD-CT-2004-506359. The aim of this part of the work was to gain more knowledge on the mechanism and kinetics of iron induced phospholipid oxidation in liposomes. The continuous measurement of oxygen uptake was chosen as the main method for these studies.

More specifically, the work was focussed on the following questions:

- the mechanism of iron induced phospholipid oxidation in liposomes
- how factors such as:
    - temperature,
    - amount of injected Fe^{2+},
    - lipid concentration,
    - pH,
    - different salts

influence lipid oxidation rate

- the effect of pH and salts on liposome Zeta potential and lipid oxidation induced by Fe^{2+}
- development of a robust and validated mathematical model for oxygen uptake measurements which could be valuable at conditions that differ from experimental ones used to establish the model.

3. METHODOLOGY

The general descriptions of the methods used in the studies are given in the papers. This section will provide a short background of the methods and the chosen conditions.

3.1. Phospholipid isolation

The marine phospholipids that were used to study lipid oxidation pathways and kinetics in liposomes were isolated from cod roe. The extraction of total lipids was performed according to the method of Bligh and Dyer (1959). Phospholipids were isolated from total lipids using the acetone precipitation method as described by Kates (1991), with a few modifications described in paper I (Figure 4). In the literature several column chromatography methods (absorption (solid-liquid), liquid – liquid partition, ion-exchange) that could be used for polar lipid separation have been reported (Kates, 1991). These methods are however complicated to perform and adaption based on raw material is usually required. The acetone precipitation method was chosen because it does not require special equipment and it is very simple. It is based on the insolubility of phospholipids in cold acetone. Phospholipids precipitate and were collected. Isolated phospholipids were stored in chloroform under nitrogen at – 20^{o}C until needed.

3.2. Liposome preparation and characterisation

Liposome preparation started with solvent removal from the phospholipids by gently passing nitrogen (99.99%) over the solution. After removal of the solvent, the phospholipids were kept under vacuum for 1 hour. The dried film of phospholipids was dissolved in a 5mM pH 5.5 MES (2-morpholinoethanesulfonic acid, Sigma) buffer to a concentration of 30mg/ml.

MES buffer is known as a “Good type” buffer and it was chosen for several reasons. It does not bind iron as for example phosphate buffers (Kuzuya et al., 1991, Djuric et al., 2001) and has a very low solubility in nonpolar solvents. As most of our oxidation experiments were performed at pH ≈5.5 (observed highest oxidation rate), MES buffer was also a good candidate as the effective pH is given as 5.5-6.4 (pK=6.1).

To get phospholipids dispersed in aqueous media as bilayer structured liposomes several methods such as hand shaking, sonication, membrane extrusion etc. can be used (New, 1990). The probe sonication method was chosen because only a short time was

needed to disperse phospholipids in the buffer. The solution was sonicated for 15s x 10 times with an MSE Ultrasonic Disintegrator Mk2 (MSE Scientific instruments, Sussex, England). Each sonication treatment was followed by a 30s break and cooling in ice was used to avoid temperature increase. A total sonication time of 2.5 min was enough to disperse all phospholipids in buffer. When sonication time of 5 min was used to make liposomes, no decrease in particle size distribution was observed. According to the literature increased sonication time decrease oxidative stability of liposomes (Klein, 1970, Genot et al., 1999). Due to this a sonication time of 2.5 min was chosen as the optimal time.

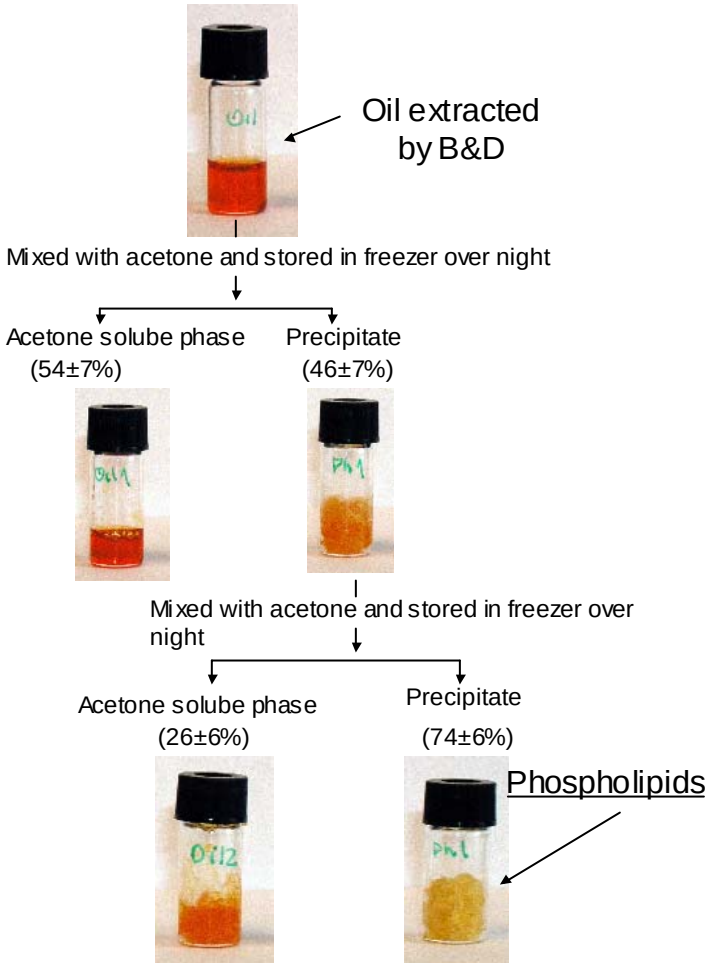


Figure 4. Phospholipid isolation from total lipid extracted from cod roe.

The particle size distribution and the Zeta potential of the liposomes was analysed with a Zetasizer Nano ZS (Malvern instruments Ltd, Worcestershire, UK). The measurements were done at a phospholipid concentration of 6mg/ml. Based on the measured intensity distribution of scattered light from the liposomes the number (D, nm)

mean sizes of the liposomes was determined. Three replicate analyses were performed and the results were expressed in diameter (nm) and intensity (%) as mean values. The Zeta potential (mV) of liposomes was determined from electrophoretic mobility measurements.

3.3. Oxidation experiments

The consumption of dissolved oxygen by liposomes in a closed, stirred, water jacketed cell, was used as a measure of the rate of lipid oxidation. The concentration of dissolved oxygen was measured continuously by a polarographic oxygen electrode (Hansatech Instrument Ltd, Norfolk, UK).

Polarographic oxygen electrode (Figure 5) consists of an electrode unit (a) mounted on a control unit (b). The electrode disk (c) forms the floor of the reaction cell (d) where liposomes or other reaction mixtures could be added. Magnet (e) mixes the reaction mixture, to maintain the same oxygen concentration throughout the whole volume. Plunger (f) prevents oxygen diffusion from the atmosphere. The capillary hole in the plunger (g) enables injection of reactants during the measurement. The reaction cell is water jacketed so that it was possible to perform oxidation experiments at different temperatures. The temperature range used in the studies was 20^{o}C-40^{o}C.

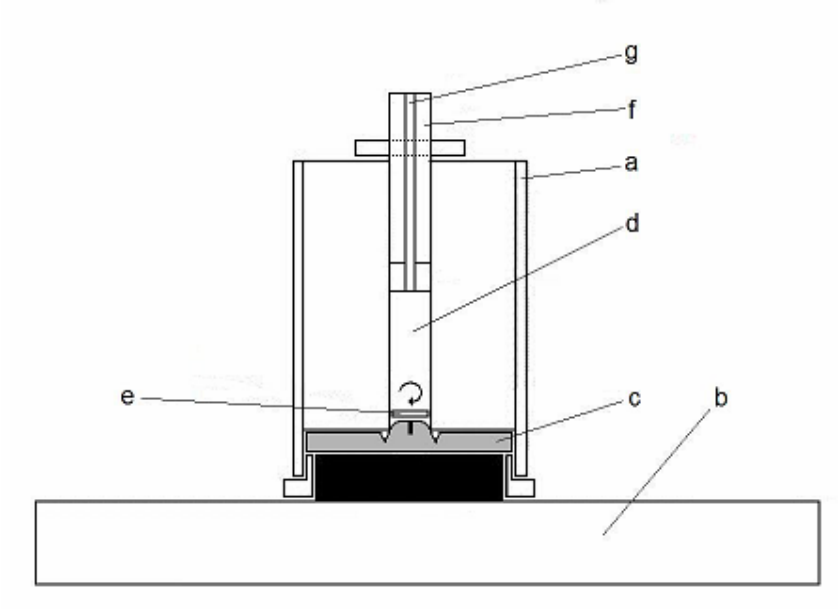


Figure 5. Schematic presentation of the oxygraph.

The oxygen electrode consists of two electrodes: a platinium catode and a silver anode. An electrolyte bridge between the electrodes is established by placing a small

amount of electrolyte solution on the dome area of the electrode which is held by a paper spacer and a polytetrafluoroethylene membrane. Application of a stable polarizing voltage across the electrodes from the electrode control box results in ionization of the electrolyte and a flow of current through the electrolyte. The magnitude of this current flow is proportional to the concentration of oxygen dissolved in the electrolyte which in turn is proportional to the concentration of oxygen in the surrounding media (solution in the reaction cell).

The Sensitivity of the dissolved oxygen concentration measurements by oxygen electrode was analysed using the following reaction which generates known quantities of oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The sensitivity experiment was performed as described below. An aliquot of known concentration of a peroxide solution was added to 1ml of nitrogen bubbled MES buffer with a dissolved oxygen concentration of approx. 50µM. The concentration of peroxide stock solution was checked using a molar extinction coefficient of H_2O_2 of 43.6 $M^{-1}cm^{-1}$ at 240nm (Banerjee, 2002). Changes in dissolved oxygen concentration were measured for a few minutes, after this the enzyme peroxidase was added. The oxygen concentration generated ($\Delta C(O_2)$) was calculated from the following equation:

$$\Delta C(O_2) = C(O_2)_2 - C(O_2)_1$$

Where: $C(O_2)_2$ is the concentration of dissolved oxygen obtained after addition of enzyme solution after reaching stable conditions; $C(O_2)_1$ is concentration of dissolved oxygen when peroxide was added to the buffer (Figure 6).

The measured generated oxygen amount was similar to the calculated oxygen amount when different concentrations of hydrogen peroxide was added (Figure 7) which proved the sensitivity of the measurements.

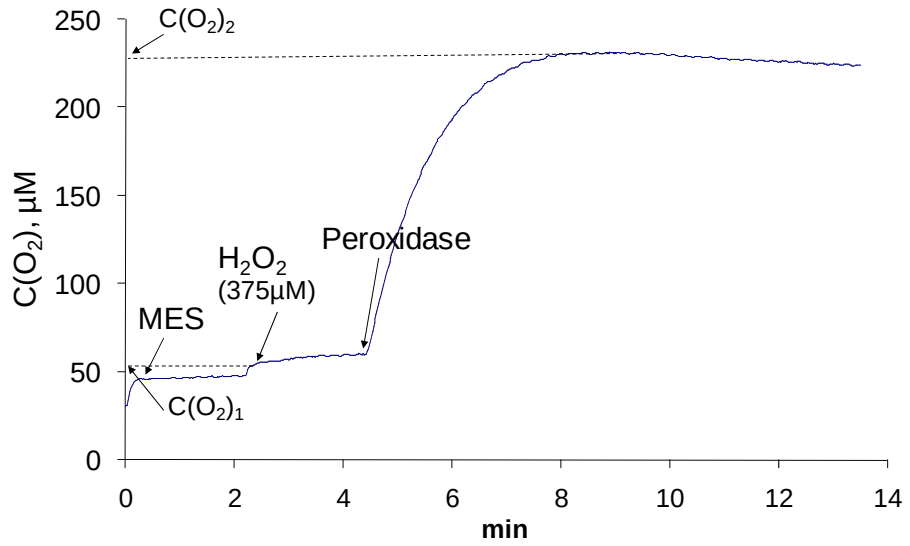


Figure 6. Dissolved oxygen concentration changes in buffer after adding H_2O_2 and peroxidase enzyme.

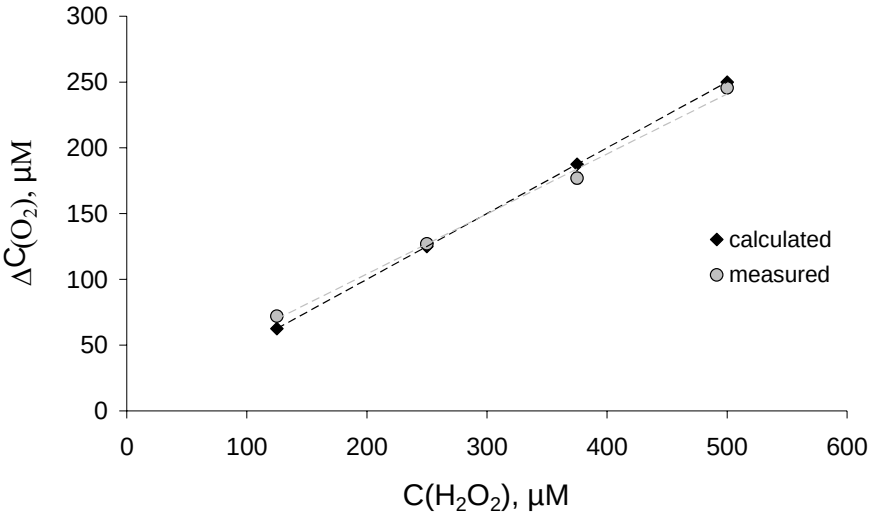


Figure 7. Measured and calculated concentrations of generated oxygen after from different concentration of hydrogen peroxide in the buffer peroxidase solution.

pH and salt . pH were adjusted by replacing some of MES buffer, which was used to dilute the 3% liposome solution, with 0.1N NaOH, 0.01N or 0.05N HCl and the pH was verified after the oxidation experiment. Different salt concentrations in the liposome solutions were made by replacing some MES buffer used for liposome dilution with salts solutions in MES buffer.

Fe^{2+} solution. The solubility of Fe^{2+} is higher at lower pH (Graf et al., 1984). Due to autooxidation of Fe^{2+} at higher pH (Kawakatsu et al, 1984), the stock solutions of

15mM $FeSO_4 \cdot 7H_2O$ (99.5%, Merck) was made in acidic solution. The working solution (0.375mM Fe^{2+} in 5mM MES-buffer, pH 5.5) was prepared daily. The pH of the final liposome solution was more affected when iron stock solution was made in 1N HCl acid. In the studies described in paper II, the stock solution was prepared in 0.5N HCl to avoid a relatively large decrease in pH in the final liposome solution.

Oxygen uptake rate (OUR). When measuring dissolved oxygen concentration in liposomes, background oxygen uptake rate was observed for 4-6min before Fe^{2+} injection. After injection of Fe^{2+} into the system, a fast decrease in dissolved oxygen was observed. This was followed by a slower linear decrease in oxygen, from which the oxidation rate was found by subtracting the background oxygen uptake rate. In paper I the slope of linear dissolved oxygen decrease was calculated with the SigmaPlot8.0 program, where the experimental values were fitted to the equation:

$$y=A + ae^{-bt} + ct$$

where y is oxygen uptake rate; A, a, b, c are constants and t is time. As most of our studies were based on the rate of slower linear decrease, in paper II and paper III the OUR of slower linear decrease was calculated using Oxygraph software "oxyg32". Rate calculation function calculates the rate between a pair of user-define Start and End points. The time period was positioned by two red triangular tags as shown in Figure 8 for oxygen uptake calculation by the software. The oxygen uptake rate (r) was calculated subtracting the background rate:

$$r= r_2-r_1$$

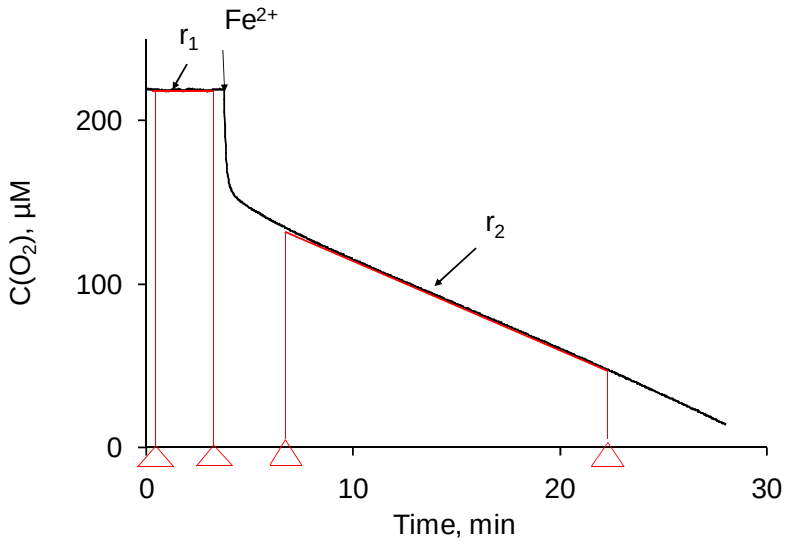


Figure 8. Oxygen uptake rate measurement by polarographic oxygen electrode

4. RESULTS AND DISCUSSION

4.1. Marine phospholipids from cod roe

Seven different isolations of marine phospholipids from cod roe were used in the studies. Thin layer chromatography (TLC) analyses showed that total lipids extracted from cod roe contained 84-86% of phospholipids. Phospholipids isolated by the acetone precipitation method contained 96 - 99.4% phospholipids. Qualitative C-13 NMR analysis was performed for additional analysis of lipid class composition. From the comparison of the intensity of the CH signals from the sn-2 (peak 9) and sn-1,3 (peak 4) signals (Figure 9), it can be seen that isolated phospholipids contained reduced amounts of triacylglycerols. The intensity of the peaks assigned as carbons of cholesterol (peaks 2 and 11, Figure 9) also decreased in isolated phospholipids compared to the total fat extracted from cod roe (Figure 9). When studying the glycerol part of the NMR spectra, it is also seen that the phospholipids consisted of mainly phosphatidylcholine and phosphatidylethanolamine.

Cod roe can contain about 0.7ppm on carotenoids on a wet weight basis (Grung et al., 1993). For measurement of total carotenoid amount in isolated phospholipids, small amounts of phospholipids were dissolved in hexane and total carotenoid was measured spectrophotometrically at 470nm using the $E_{1\%}$=2100 (Tolasa et al., 2005). All isolations of phospholipids contained <0.001% of carotenoids.

The fatty acid composition was determined by GC of methyl esters prepared by base catalysed transesterification. In average all the isolated phospholipids (n=7) contained 30 ± 4% saturated, 29 ± 5% monounsaturated and 41 ± 9 % polyunsaturated fatty acids. DHA made up 26 ± 9% and EPA made up 11 ± 2% of total fatty acids.

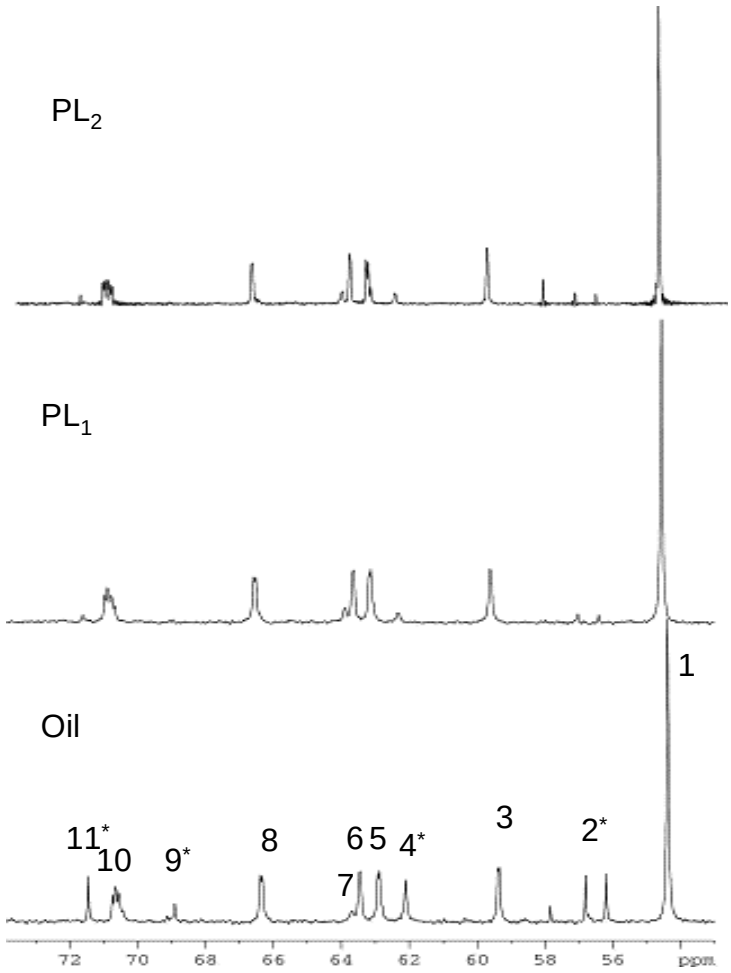


Figure 9. C-13 NMR of glycerol region of total lipids extracted from cod roe (Oil) and two separate phospholipid isolations (PL_1 and PL_1). Peaks were assigned as following: 1- $N(CH_3)_3$ PC, 2 – cholesterol, 3 CH_2N PC, 4 – TAG (sn 1,3), 5 – sn1 (PE/PC), 6 – sn -3, PC, 7 – sn – 3 PE, 8 – PC – (CH_2O), 9 – TAG (sn 2), 10 – PL (sn2), 11 – cholesterol. (Peaks were assigned according to Medina and Sacchi, 1994 and Falch et al., 2006)

4.2. Oxygen uptake – a method to study lipid oxidation in liposomes

Oxygen is consumed during oxidation of lipids. Therefore, oxygen availability is an important factor for oxidation and when studying oxidation kinetics. During storage experiments the oxidation rate in food could be affected both by the rate of the lipid oxidation reactions and by the rate of oxygen diffusion. In some cases, oxygen transport (diffusion) can become a limiting factor for the rate of lipid oxidation. Oxidation of fatty acids in liposomes is expected to occur in the hydrophobic core. In the studies of Subczynski et al. (1989), an oxygen permeability coefficient of 114.2 cm/s was observed

for L-α-dioleoylphosphatidylcholine membranes at 30°C. The small cod roe phospholipid liposomes has a volume mean diameter of 46 ± 6 nm and the large particles, had a volume mean diameter of 4328 ± 300 nm (Figure 10). Due to this, the thickness of the liposome membrane has to be less than ~20nm for the small particles and ~2000nm for the large ones (radius of droplet). The time for an oxygen molecule to cross the membrane should then be between 2ns for the small particles and 0.2μs for the large particles. Therefore the oxygen transport from water to the hydrophobic core should not be a limiting factor for the measured rate of oxidation as the oxygen concentration was measured each second.

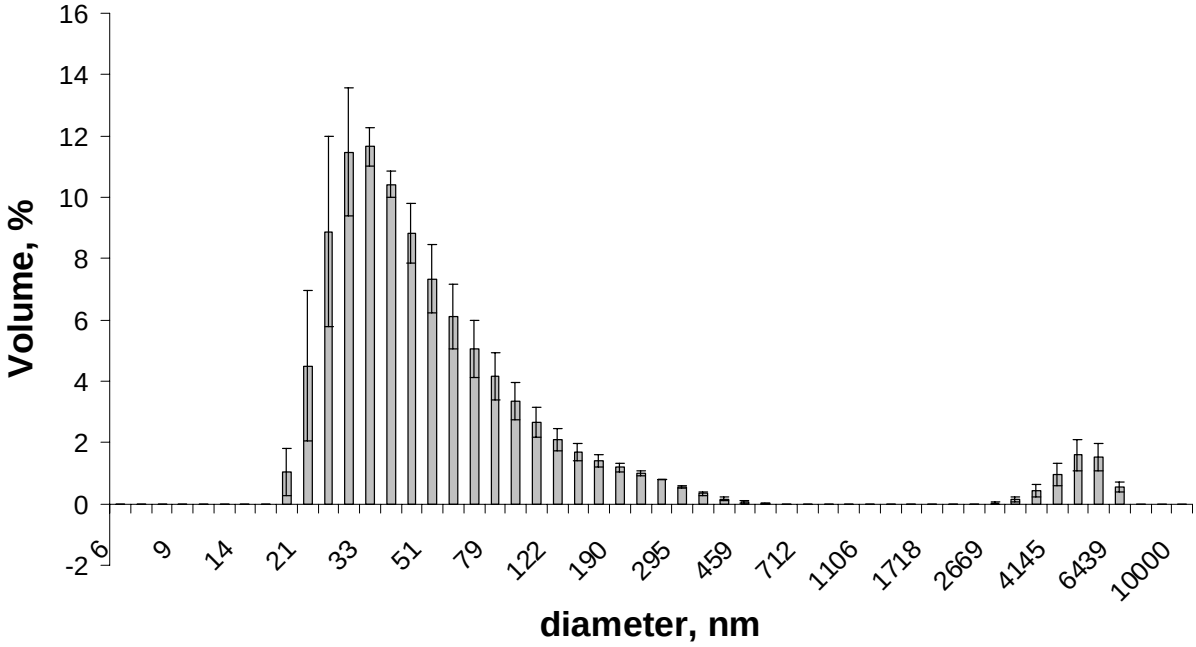


Figure 10. The particle size distribution in liposomes

4.3. Oxidation mechanism of PUFAs in liposomes (Paper II)

Both Fe^{2+} and Fe^{3+} induced lipid oxidation of cod roe phospholipids in the liposomes as PV and TBARS values increased together with oxygen consumption. When Fe^{2+} was added to liposomes to catalyse lipid oxidation, an *initial drop in concentration of dissolved oxygen* was observed, this was followed by a *slower linear decrease in concentration of dissolved oxygen* (Fig. 8). However, when Fe^{3+} was added to the liposome solution, only the slow linear decrease in concentration of dissolved oxygen

was observed. The initial drop was hardly detectable. In oxidation studies of phospholipid emulsions by ferrous and ferric ions, Kawakatsu et al. (1984), observed a similar type of changes in formation of conjugated dienes. Genot et al. (1994) also observed an initial (faster) drop followed by a slower decrease in dissolved oxygen after addition of Fe^{2+} - ascorbate mixture to liposomes.

4.3.1. Initial drop in dissolved oxygen

The initial drop in dissolved oxygen was proportional to the added Fe^{2+} concentration being 5.6 ± 0.5 times higher than the amount of added iron. The rate of the initial drop in dissolved oxygen was also dependent on phospholipid concentration in liposome solution being slower at the lower phospholipid concentration. Addition of triphenylphosphine (TPP), which breaks down peroxides to alcohols (Fukuzawa et al, 1993), reduced both initial drop and slower oxygen uptake. Addition of the peroxy radical scavenger, N, N'-diphenyl-p-phenylene-diamine (DPPD) (Noguchi et al., 1998), also reduced uptake of oxygen. From the observations described above it was concluded that after addition of Fe^{2+} to liposomes, the fast decrease in Fe^{2+} concentration during the initial oxygen uptake phase was due to oxidation of Fe^{2+} to Fe^{3+}. As oxidation rate of Fe^{2+} via peroxide breakdown reaction was observed to be 79μM/min, the alkoxy radical formation rate was also supposed to be 79μM/min. One alkoxy radical further propagated lipid peroxidation by chain branching leading to production of several peroxides accompanied by oxygen consumption as a rate of 258 μM O_2/min was measured during the initial drop in oxygen. When equilibrium between Fe^{2+} and Fe^{3+} was achieved after the initial fast oxygen uptake, a constant rate of oxygen uptake was observed. A decrease in oxidation rate when Fe^{2+} was oxidised to Fe^{3+} was also observed by Yoshida and Niki (1992), but in their studies the decrease was not quantified.

4.3.2. Constant oxygen uptake

When EDTA was added at double the concentration of Fe^{2+}, both the initial and the constant oxygen uptake was immediately stopped (Fig. 11). This indicates that iron ions were involved in oxidation reactions and continuously producing radicals resulting in oxidation, measured as the initial drop in dissolved oxygen and the constant oxygen uptake.

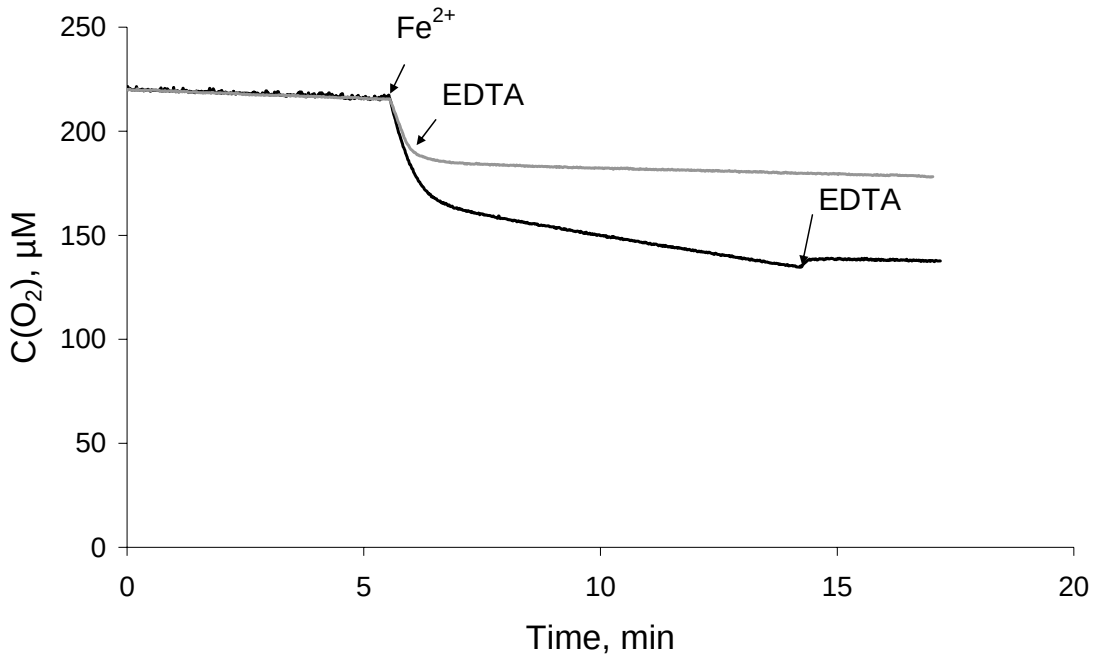


Figure 11. 15µM EDTA influence on the initial and linear oxygen uptake phase induced by 7.5µM Fe^{2+}. Phospholipid concentration in the liposome solution was 0.3mg/mL.

The formation of Fe^{2+} from Fe^{3+} was also observed and most probably occurred due to peroxide breakdown by Fe^{3+} producing peroxy radicals (reaction 1 in Background). Reaction of Fe^{3+} with peroxides was slower (~0.25 µM Fe^{3+}/min) than the oxidation of Fe^{2+} by peroxides measured during the initial drop. The formed Fe^{2+} further reacts with peroxides producing alkoxy radicals. This leads to a redox cycling between Fe^{2+} and Fe^{3+} in the *radical generating cycle* (Figure 12) where the reduction of Fe^{3+} becomes a limiting factor. When equilibrium is established between Fe^{2+} and Fe^{3+} the slower constant oxygen uptake is observed. At equilibrium, the concentration of Fe^{2+} was found to be less than 1/10 of the Fe^{3+} concentration. The rate of radical production in the *radical generating cycle* can be calculated to 0.5 µM/min based on equal oxidation and reduction rates of the iron species. Both peroxy and alkoxy radicals formed in the radical generating cycle are proposed to subtract bisallylic hydrogen from fatty acids (Frankel, 2005). The generated lipid radical (L·) take part in the *peroxide production cycle* leading to formation of several peroxides as shown in the lower cycle in Figure 12. The oxygen consumption at constant oxygen uptake was observed to be 4-6 µM O_2/min. Constant oxygen uptake rate has been shown to be independent of the concentration of dissolved oxygen from 100 % saturation down to at least 5% (paper I). To achieve constant oxygen uptake rate, the concentration of radicals, in specific lipid radicals (L·), must be constant.

Their concentrations are regulated by the production in the radical generating cycle and removal by the termination reactions. As the radical concentrations are constant when the oxidation rate is constant, the production rate and the termination rate should be equal. Based on these assumptions it is possible to calculate that the peroxy- and lipid radicals in average would react approx. 10 times in the peroxide producing cycle before they are removed by termination reactions.

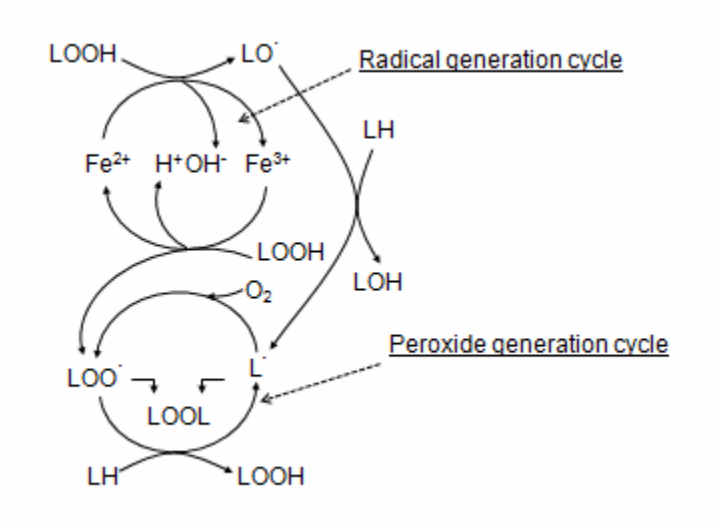


Figure 12. Proposed mechanism for oxidation of unsaturated fatty acids (paper II).

According to our proposed theory, the linear uptake of oxygen when adding Fe^{2+} or Fe^{3+} should be the same. However, the difference in solubility of Fe^{2+} or Fe^{3+} hydroxides was observed to play a role in determining the concentration of active iron on the surface of the liposomes. The lower solubility of Fe^{3+} hydroxides caused the slightly slower linear oxygen uptake.

4.4. Factors that influence lipid oxidation (Paper I, II, III)

The slower constant oxygen uptake was not influenced by dissolved oxygen concentration as the oxygen uptake observed after fast initial drop in dissolved oxygen followed the linear function till approx. 5 μM.

4.4.1. Temperature (paper I)

The rate of the *slower linear decrease in concentration of dissolved oxygen* was used to study the effect of temperature on oxidation of phospholipids. Oxygen uptake rate (r) was measured at different temperatures (20, 25, 30, 35 and 40°C) and the effect on the

rate of oxygen uptake was analysed in terms of the Arrhenius activation energy (E_a). The logarithmic function of oxygen uptake rate vs reciprocal absolute temperature gave a straight line, when using different liposome concentrations (3-12mg/ml) and concentrations of iron (7.5 and 11.25uM), indicating that the oxidation mechanism was the same in this temperature range. The calculated activation energy E_a was in the range 60-86 kJ/mol. Higher activation energy implies that a smaller temperature change is needed to induce a certain change in the rate of oxidation (Tan et al., 2001). The E_a found in our experiments was in agreement with metal reduced lipid oxidation activation energy in literature given down to 62-105 kJ/mol (Labuza, 1971) and free radical lipid oxidation activation energy of 62-105 kJ/mol (Labuza, 1984).

In paper II it was observed that the reduction of Fe^{3+} back to Fe^{2+} has a low activation energy that indicates that it might be a transport limited process (Levenspiel, 1972). However, for the constant oxygen uptake the activation energy was much higher 60-86 kJ/mol, indicating that the process was not limited by oxygen transport.

4.4.2. Fe^{2+} concentration (paper I)

A linear relationship between the concentration of Fe^{2+} and the rate of constant oxygen uptake was observed below the critical ratio between Fe^{2+} and lipid (10 μmoles Fe^{2+}/g lipids). The lipid oxidation may be determined by the concentration of iron bound to the liposome surface rather than by the average iron concentration in the solution. It could be that there is a "critical" concentration of Fe^{2+}, which can be bound to the liposome surface and determine lipid oxidation rate (Paper I). According the proposed model (Figure 12), higher added concentration of Fe^{2+} to liposomes should increase the constant oxygen uptake rate as the substrate concentration for the radical generation cycle increases.

4.4.3. Lipid concentration (paper I and II)

To be able to measure the oxygen uptake rate during the initial drop, the phospholipid concentration in the liposome solution had to be lowered. The rate of oxygen consumption during the initial drop was dependent on the phospholipid concentration in liposome solution. When the concentration of the phospholipids in liposome solution was reduced, the rate was lower (Paper II). The peroxides are the proposed substrates for initiation of iron induced lipid oxidation according mechanism

given in Figure 12. At the initiation of oxidation, in a liposome solution with low phospholipid concentration, the concentration of peroxide (substrate for reaction 1) and/or allylic center of unsaturated fatty acids available per Fe^{2+} unit probably became limiting, resulting in a lower oxidation rate of phospholipids and Fe^{2+}. A linear relationship between oxygen uptake rate and lipid concentration in the liposome solution was observed, when the lipid concentration varied between 1.5-15 mg/ml and the Fe^{2+} concentration was 7.5 and 11.25 μM. However, it is not the total fat content, but the amount of unsaturated fatty acid moieties, that is important for lipid oxidation (Labuza, 1971). A significant linear relationship ($p<0.1$) between concentration of double bonds in PUFAs and oxygen uptake rate was also observed when the injected Fe^{2+} concentration was 7.5 and 11.25 μM.

4.4.4. pH (paper I and III)

The effect of pH on lipid oxidation is associated with the availability of metals and the surface activity (ionisation of charged surface molecules) (Nawar, 1996). The influence of pH and the linear oxidation uptake was studied and a bell shaped relation between pH and the rate of the linear oxygen uptake was observed. Changing the pH of the liposome solution resulted in changes in the Zeta potential; the Zeta potential became more negative at higher pH. At pH higher than ~6 the Zeta was constant approx. -25mV.

The rate of oxygen uptake was influenced by the pH dependent Zeta potential of the liposomes. The oxygen uptake rate did not change significantly when the Zeta potential of the liposomes was positive. An increase in oxygen uptake rate was observed with decreasing Zeta potential at negative Zeta potentials but at Zeta potentials below -25mV, the linear oxygen uptake rate started to decrease.

The increase in oxygen uptake rate, at negative Zeta potentials, could be due to attraction of Fe^{2+} to the liposome surface. The binding of Fe^{2+} to the membrane (most probably to the phosphate group) was reported to be the first step in the iron induced lipid oxidation (Garnier – Suillerot et al., 1984). Lower Zeta potential at higher pH (paper III) could be due to conformation changes of the lipid polar group (negative phosphate group could move more to the water phase (Makino et al., 1991)) or due to increase of concentration of the negatively charged ions in the particle inside the shear plane. The lower Zeta potential could cause better attraction of positive iron ions to the surface and due to this better iron interaction with hydroperoxides and higher oxidation rate. Fukuzawa and Fujii (1992) found that addition of free Fe^{2+} to negatively charged SDS

(sodium dodecylsulfate) micelles in the presence of linoleic acid hydroperoxide resulted in peroxidation of linoleic acid. Fe^{2+} was not attracted to positively charged surface of the TTAB (tetradecyltrimethylammonium bromide) micelles, and consequently peroxidation was not initiated.

When the pH of the liposome solution was reduced from 5.5 to 7, the Zeta potential was approx. -25mV. The effect of pH in this pH range might be attributed to the autoxidation of ferrous ions to ferric ions which in alkaline pH could be hydrated, becoming ferric hydroxide, which has less catalytic activity (Kawakatsu et al, 1984). Tadolini and Hakim (1996) and Welch et al. (2002) observed that when the pH of the buffer increased from 6.5 to 7, the rate of Fe^{2+} autoxidation increased.

Linear oxygen uptake was probably affected both by the influence of pH on the liposome surface and the availability of iron.

The amount of oxygen consumed during the initial fast drop was dependent on the pH of the liposome solution, which was higher at lower pH (data not presented). The smaller fast drop in dissolved oxygen concentration at pH 6-7 in liposomes could be explained by autooxidation of Fe^{2+} to ferric ion, (Kawakatsu et al, 1984). Autoxidation of Fe^{2+} (Kawakatsu et al, 1984, Tadolini and Hakim, 1996) and reduced solubility by increasing pH (Graf et al., 1984) reduced the Fe^{2+} amount that took part in the proposed radical generating cycle (Figure 12) and caused the reduced height in initial drop of oxygen. Thus, the height of the fast drop of oxygen may be attributed to the stability of ferrous ions.

4.4.5. Salts

Salts are common constituents in food and the influence on lipid oxidation is still not clear. The influence of different salts on the oxidation of liposomes was studied in paper III. Oxidation experiments with different salts (NaCl, KCl, $NaNO_3$, $CaCl_2$, Na_2SO_4, K_2SO_4, $Ca(NO_3)_2$, $Mg(NO_3)_2$ and NaH_2PO_4) were performed while keeping the same ionic strength in the liposome solution. However, the addition of Na_2SO_4, K_2SO_4, $NaNO_3$, $Ca(NO_3)_2$, $Mg(NO_3)_2$ did not change the oxidation rate significantly, indicating that neither the used cations (Na^+, K^+, Ca^+, Mg^+) nor SO_4^{2-} and NO_3^- influenced oxidation rate. Oxidation rate was reduced by increasing ionic strength when NaCl, KCl, $CaCl_2$ and NaH_2PO_4 was added, indicating that chlorides and dihydrogen phosphate were the active ions in reducing oxidation rate. The phosphate was more effective in reducing oxidation

rate than chlorides. The inhibition of Fe^{2+} induced oxidation of liposomes by phosphate might be due to the phosphate chelation of iron (Djuric et al., 2001, Kuzuya et al., 1991). The relationship between oxygen uptake and three chloride sources was the same when the oxygen uptake was related to the chloride concentration. Mabrouk and Dugan (1960) explained the inhibition effect of NaCl on oxygen uptake rate in emulsions by decreased solubility of oxygen. However, in our studies, the oxygen uptake rate was constant at oxygen concentrations between 200 – 5 μM (paper I), and the reduction in the concentration of dissolved oxygen at 0.15 M NaCl is only 5%. (Doran, 1995). Mei et al. (1998a) proposed that the ability of NaCl to decrease oxidation, at low iron concentration, could be due to the ability of NaCl to remove iron from the emulsion surface. This explanation does not fit our observation as none of the tested cations influenced the oxidation rate. Osinchak et al. (1992) found that substituting potassium with sodium had little effect on oxidation of liposomes, but a stimulatory effect of both sulfate and chloride salts on oxidation of liposomes was observed. As salts has been found to act both as prooxidants and antioxidants, their effect could also be dependent on the nature of the systems involved (McClements and Decker, 2000)

When liposomes contained different phosphate and chloride concentrations, a reduction in oxygen uptake rate was also observed. This reduction in oxygen uptake rate was only dependent upon the dihydrogen phosphate concentration, when the relative value of oxygen uptake rates are plotted for different chloride concentrations. This relationship indicates that phosphate and chlorides has an negative additive effect on oxidation in liposomes and that these two anions have different modes of action.

The increase in Zeta potential by adding salt could be due to more tightly compacting of the counterions near the droplets, resulting in a greater shielding of the surface charge (Hunter, 1993). Magnesium and calcium salts resulted in positive Zeta potentials. With addition of one of the following salts, $CaCl_2$, NaCl and KCl, a linear relationship between the Zeta potential and the oxygen uptake rate was also observed. The linear oxygen uptake rate decreased with increasing Zeta potential (more salt added). However, reduction of oxygen uptake rate by phosphate was found not to be related to the Zeta potential of the liposomes. In our studies, addition of salts increased the Zeta potential, which indicates that the counterions are more tightly compacted near the droplets (Mei et al., 1998b) or that the conformation of the polar groups of the phospholipids was changed, with the positively charged group being closer to the surface (Makino et al., 1991). However, only chlorides and phosphates influenced the oxidation.

4.5. Robustness of oxygen uptake rate measurements (Paper IV)

The main goal of this study was to gain insight into how various factors interact and influence the response, and how predicted (modelled) values fit to observed values. The variability in the measurements was also of interest.

The effect of five variables on the rate of lipid oxidation was investigated using experimental design and response surface modelling. The central composite design was based on the five design variables with three levels: pH (4.5; 5.5; 6.5), ionic strength adding NaCl (0; 0.5; 1 M), Fe^{2+} (5; 10; 15 μM) and lipid (0.2; 0.7; 1.2) concentration and temperature (30; 35; 40^{o}C). The maximum response level was observed at the highest temperature, Fe^{2+} and lipid concentration and the lowest ionic strength and pH.

For signal to noise ratio (SNR) studies the three replicates were investigated for each experimental setting. The standard deviation was observed to be around 10% of the measured value. When SNR was plotted against the mean response it was observed to be the same in the entire measured range, indicating that the measurement error of the system is proportional to the signal level.

The predicted values were calculated based on the data described in paper I. The response surface model fitted reasonably well for the lower values, but slightly underestimated the higher values.

Different isolations of phospholipids were used for each experimental design (total 3). The models that were fitted to data from one experimental design still gave fairly good prediction when applied to new data generated from another isolation of phospholipids.

4.6. Mass spectrometry analyses for oxidation in liposomes

Polar head groups of phospholipid can be ionised and carry a charge. Due to this liposomes can be analysed by mass spectrometry. Electron spray liquid chromatography mass spectrometry (LC-MS) and electron spray injection time-of-flight (ESI TOF-MS) mass spectrometry were tried as advanced tools to study oxidation in liposomes. Analyses were performed by operating in positive and negative ion mode. Positive and negative ion spray ionization mass spectrometry was used to obtain lipid profile of vesicles. Part of spectra observed in positive and negative modes from ESI-TOF-MS are given in Figure 13 with peaks assignment in table. 2. Accurate mass to charge ratios (m/z) were obtained from ESI-TOF-MS. The assignment of the peaks was done by comparing masses of different fatty acids obtained from GC analyses with phosphatidylcholine or

phosphatidylethanolamine groups. The peaks obtained in negative mode and assigned as PC were calculated taking into account that the positive choline group was neutralizes by negative charged acetate group (~58). For molecular weight calculation masses of the different elements occurring in phospholipids were taken from Schiller et al. (2004).

When LC-MS analyses were used to study lipid profile changes during oxidation, the peaks obtained from LC-MS were assigned according to the peaks obtained from ESI-TOF-MS (that gives more accurate results).

Oxidation experiments in mass spectroscopy injection vials were performed as follows. The vial, with a glass ball inside to facilitate mixing, was completely filled with liposome solution. To catalyse the lipid oxidation, Fe^{2+} working solution with a final Fe^{2+} concentration of 7.5 μM in the liposome solution was added. At time points between 0-120 min 20 μl aliquots of the liposome solution were injected to the mass spectrometer for analysis. The time difference between the two samplings for negative modes (and positive modes) was approx. 10 min. The changes in abundance were plotted vs time (Figure 13 and 14). During oxidation the native ion signals assigned as PC with polyunsaturated fatty acids decreased both in positive and negative modes. The decrease in abundance was probably due to oxidation of polyunsaturated fatty acids, as the abundance of peaks assigned as PC with saturated and monounsaturated fatty acids did not change. Appearance of some peaks was observed at higher m/z region, but due to low abundance they were difficult to identify.

LC-MS analyses can be used to detect oxidation products of phospholipids, however some improvements in methodology should be done. MES buffer itself gave peaks with m/z 194, 389, 411, 628, 845 in negative mode and m/z 196, 218, 413 etc in positive mode. High abundance of the peaks made analyses complicated. Different buffers should probably be investigated.

b) Positive mode

a) Negative mode

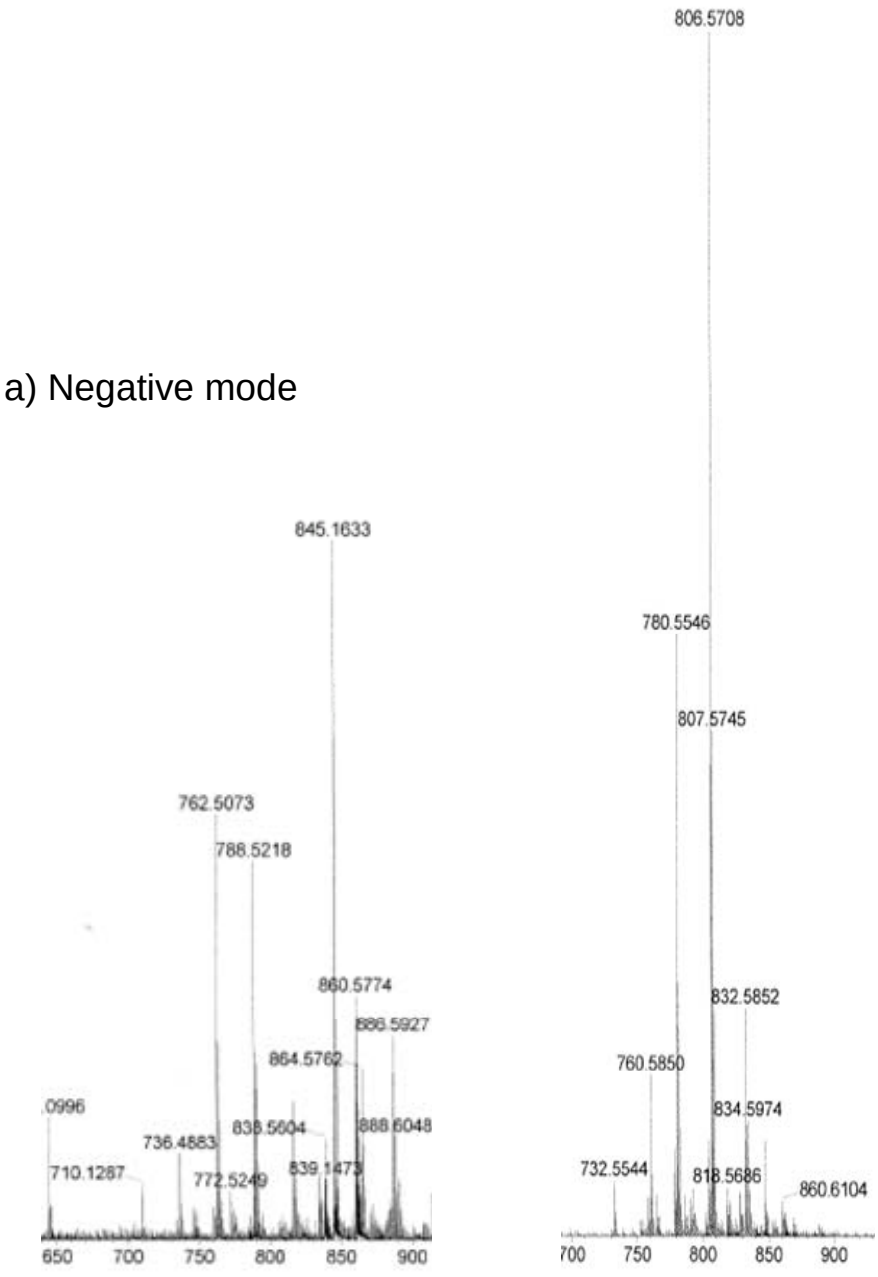


Figure 13. ESI-TOF MS spectra of phospholipids liposomes from: a) negative mode b) positive mode analyses. Assignment of the peaks is given in Table 2.

Table 2. Lipid profile of vesicles. Mass measurements with LC-MS, ESI-TOF-MS and calculated.

m/z				Composition		
LC-MS	ESI-TOF	Calculated	Mode	PL	Possible fatty acid composition	
732.8	732.5544	732.5543	positive	PC	16+16:1	
760.8	760.5850	760.5856	positive	PC	16+18:1	16:1+18
780.7	780.5546	780.5543	positive	PC	18:1+18:3	16+20:5 14+22:5
806.8	806.5708	806.5700	positive	PC	16+22:6	18:1+20:5
832.8	832.5852	832.5856	positive	PC	18:1+22:6	
736.7	736.4883	736.4917	negative	PE	16+20:5	14+22:5
762.8	762.5073	762.5074	negative	PE	16+22:6	18:1+20:5 16:1+22:5
788.8	788.5218	788.5230	negative	PE	18:1+22:6	
818.8	818.5906	818.5911	negative	PC (760+58)*	16+18:1	16:1+18
838.8	838.5604	838.5598	negative	PC (780+58)*	18:1+18:3	16+20:5 14+22:5
864.8	864.5762	864.5755	negative	PC(806+58)*	16+22:6	18:1+20:5
890.8	890.5923	890.5911	negative	PC (832+58)*	18:1+22:6	

m/z – ion mass divided by the charge state of ion. * - ion mass plus 58 – acetic group derivative attached to phospholipid molecule

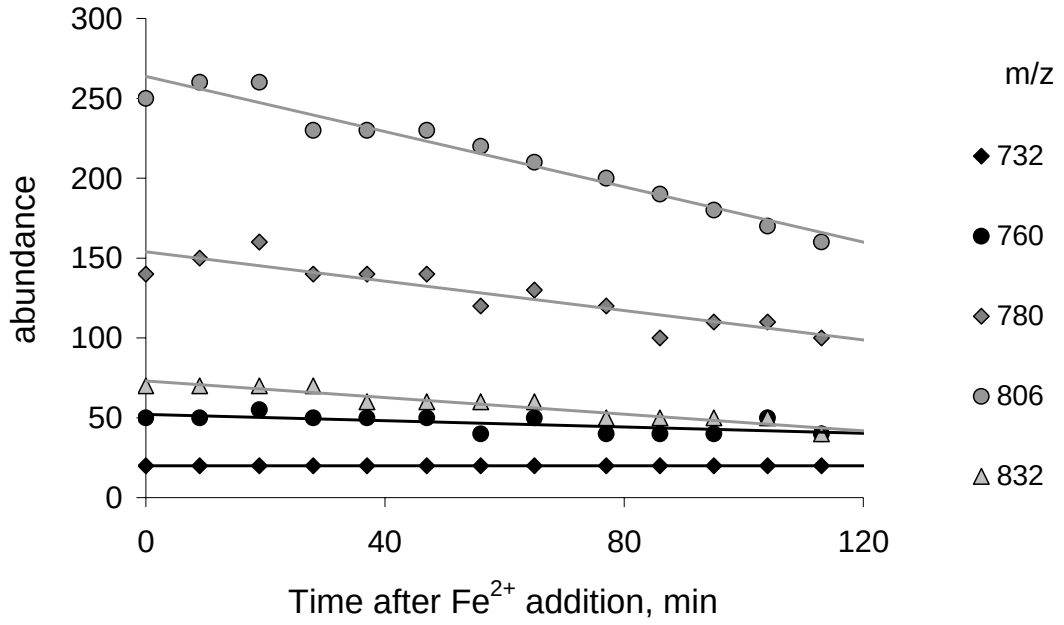


Figure 14. Changes in abundance in positive ion electronspray mass spectra as a function of time after Fe^{2+} addition to the liposomes. Phospholipid concentration in liposomes was 0.3 mg/ml, and 7.5 μM of Fe^{2+} was used to catalyse oxidation. The m/z (molecular mass divided by charge) assignment of the peaks are given in Table 2.

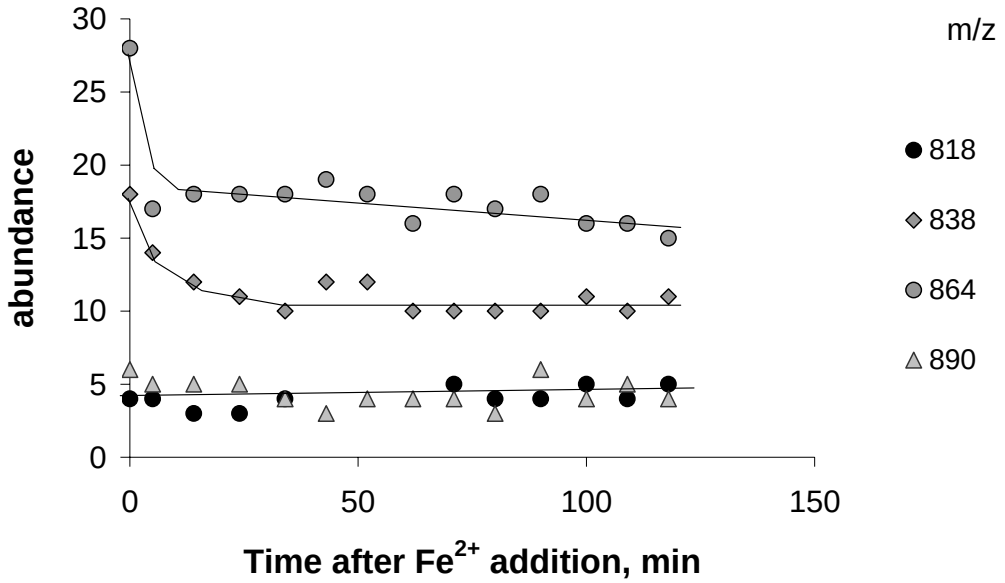


Figure 15. Changes in abundance in negative ion electronspray mass spectra as a function of time after Fe^{2+} addition to the liposomes. Phospholipid concentration in liposomes was 0.3 mg/ml, and 7.5 μM of Fe^{2+} was used to catalyse oxidation. The assignment of the peaks m/z are given in Table 2.

5. CONCLUDING REMARKS

The relationship between consumed oxygen and amount of peroxides (PV) and thiobarbituric reactive substances (TBARS) formed, indicated that both Fe^{2+} and Fe^{3+} catalysed lipid oxidation in marine phospholipids liposomes. When Fe^{2+} was added to liposomes at a concentration of approx. 10 μM, an initial drop in dissolved oxygen (oxygen uptake rate >258μM/min), followed by a slower linear oxygen uptake (oxygen uptake rate 4-6μM/min), was observed. Addition of Fe^{3+} induced only the linear oxygen uptake. The initial fast drop in dissolved oxygen was due to oxidation of Fe^{2+} to Fe^{3+} by lipid peroxides (rate of 79 μM Fe^{2+}/min). Fe^{3+} is reduced by peroxides to Fe^{2+} at a slow rate (0.25μM of Fe^{3+}/min at 30°C). When equilibrium between Fe^{2+} and Fe^{3+} was achieved, the linear oxygen uptake was observed and Fe^{3+} became the rate limiting factor in the circulation between Fe^{3+} and Fe^{2+}. Both alkoxy and peroxy radicals are presumably formed by breakdown of peroxides by Fe^{2+} and Fe^{3+}. These radicals react with fatty acids giving a lipid radical reacting with oxygen.

The oxygen consumption rate followed Arrhenius kinetics and the variation in activation energy found (60 – 87 kJ/moles*K) might be due to variations in the composition of raw materials used in the experiments and different susceptibility to oxidation.

The rate of slower linear oxygen uptake in liposomes was proportional to the concentration of iron and the lipid concentration in the assay mixture. The oxygen consumption rate was dependent on pH with a maximum observed between pH 4 and 5. The decrease in pH was followed by an increase in Zeta potential. The oxygen uptake rate did not change significantly at different positive Zeta potentials (pH >3). When the Zeta potential was negative, the oxygen uptake rate was influenced by the Zeta potential and may also be influenced by iron solubility.

Different salts had different influence on slower linear oxygen uptake in liposomes. Cations (Na^+, K^+, Ca^+, Mg^+) did not influence the rate of oxidation in the tested range (Ionic strength (I) 0 - 0.14 M). Among the tested anions: sulphates and nitrates did not change oxygen uptake rate significantly, but chlorides (KCl, NaCl, $CaCl_2$) reduced the oxidation rate down to approximately 45 % and dihydrogen phosphate down to 14 %, when I=0.14M. The effect of Cl^- and $H_2PO_4^-$ was additive. Addition of salts increased Zeta potential of the liposomes, divalent cation salts even resulted in a positive Zeta potential. When the liposomes contained different concentrations of chlorides, a linear

relationship between oxygen uptake rate and Zeta potential was observed. When phosphate was added, the oxygen uptake rate was not related to the changes in Zeta potential.

However, absolute values of the Zeta potential alone cannot be used to predict oxidation rates.

The influence of pH, temperature, concentration of NaCl, phospholipids and Fe^{2+} on slower linear rate were described by mathematical equations. The modelled data based on these equations fitted within 10% standard deviation with observed values.

6. SUGGESTIONS FOR FURTHER STUDIES

To gain more knowledge on the mechanism and kinetics of iron induced marine phospholipid oxidation in liposomes, measurements of some other oxidation products such as alcohols, polymer substances can be investigated by using NMR or some other advanced techniques.

Marine phospholipid stabilised emulsions as model system can also be used in order to increase the knowledge on the mechanism of iron induced oxidation of polyunsaturated fatty acids.

It could also be of interest to continue the oxidation studies in liposomes by screening the influence on lipid oxidation of food ingredients such as heme iron, proteins and some natural antioxidants.

The studies on how the polar groups of the phospholipids affect the oxidation kinetics would also be interesting.

Some advanced tools as LC-MS, ES-TOF-MS could provide a more precise view on oxidation pathways and oxidation compounds which are formed during oxidation.

REFERENCES

Arnaud JP (1995) Liposomes in the Agro Food-Industry. *Agro. Food Industry Hi-Tech.* 6(5), 30-36

Arseki M, Yamamoto K, Miyashita K (2002) Oxidative stability of polyunsaturated fatty acid in phosphatidylcholine liposomes. *Biosci. Biotech. Biochem.* 66(12), 2573-2577

Aruoma OI, Halliwell B, Laughton MJ, Quinlan GJ, Gutteridge JMC (1989) The Mechanism of Initiation of Lipid-Peroxidation - Evidence against a Requirement for an Iron(II) Iron(III) Complex. *Biochem J* 258, 617-620

Azuma K, Ippoushi K, Ito H, Higashio H, Terao J (1999) Evaluation of antioxidative activity of vegetable extracts in linoleic acid emulsion and phospholipid bilayers. *J. Sci. Food Agric.* 79, 2010-2016

Banerjee D, Kumar PA, Kumar B, Madhyaaoodana UK, Nayak S, Jacob J (2002) Determination of absolute hydrogen peroxide concentration by spectrophotometer method. *Current Science* 83(10), 1193-1194

Bateman NE, Ucellini DA (1984) Effect of formulation on the bioavailability of retinol, D-α tocopherol and riboflavine. *J. Pharm. Pharmacol.* 36, 461-464

Berdanier CD (2000) Fatty acids and membrane function. In: *Fatty acids in foods and their health implications.* Ed. Chow CK. Marcel Dekker, New York, US. 569-580

Bledsoe GE, Bledsoe CD, Rasco B (2003) Caviars and fish roe products. *Crit. rev. food sci. nutr.* 43(3); 317-356

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Canadian J. Biochem.* 37, 911-917

Boissonneault GA (2000) Dietary fat, immunity, and inflammatory disease In. *Fatty acids in foods and their health implications.* Ed. Chow CK. Marcel Dekker, New York, US. 777-795

Buettner GR (1993) The pecking order of free radicals and antioxidants: lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* 300, 535-543.

Calligaris S, Nicoli MC (2006) Effect of selected ions from lyotropic series on lipid oxidation. *Food Chem.* 94, 130-134

Cansell M, Nacka F, Combe N (2003) Marine lipid-based liposomes increase *in vivo* FA bioavailability. *Lipids.* 38 (5), 551-559

Chaiyasit W, Silvestre PC, McClements DJ, Decker EA (2000) Ability of surfactant hydrophobic tail group size to alter lipid oxidation in oil-in water emulsions. *J. Agric. Food Chem.* 48, 3077-3080

Chatterjee SN, Agarwal S (1988) Liposomes as membrane model for study of lipid peroxidation. *Free Radic. Biol. Med*. 4, 51-72

Choe E, Min DB (2006) Mechanisms and factors for edible oil oxidation. *Comp. Rev. Food Sci. Food Safety* 5, 169-186

Colakoglu, A. (2007) Oxidation kinetics of soybean oil in the presence of monoolein, stearic acid and iron. *Food Chem.* 101, 724-728

Corliss GA, and Dugan JR (1970) Phospholipid oxidation in emulsions. *Lipids* 5(10), 846-853

Decker EA and Hultin HO (1992) Lipid oxidation in muscle foods via redox iron. In: Lipid oxidation in foods. Ed. Angelo A. J. St., American Chemical Society Books, Washington, D.C. 33-55

Decker EA, McClements J (2001) Transition metals and hydroperoxide interactions: an important determinant in the oxidative stability of lipid dispersions. *Inform.* 12, 251-255

Djordevic D, Cercaci L, Alamed J, McClements DJ, Decker EA (2007) Chemical and physical stability of citral and limonene in sodium dodecyl sulfate-chitosan and gum arabic-stabilized oil-in-water emulsions. *J. Agric. Food Chem.* 55, 3585-3591

Djordjevic D, McClements DJ, Decker EA (2004) Oxidative stability of whey protein-stabilized oil-in-water emulsions at pH 3: Potential omega-3 fatty acid delivery systems (Part B). *J. Food Sci*. 69(5), C356-C362

Djuric Z, Potter DW, Taffe BG, Strasburg GM (2001) Comparison of iron-catalyzed DNA and lipid oxidation. *J. Biochem. Mol. Toxicol.* 15(2), 114-119

Dobarganes MC, Velasco J (2002) Analysis of lipid hydroperoxides. *Eur. J. Lipid Sci. Technol.* 104, 420-428

Donnely JL, Decker EA, McClements DJ (1998) Iron-catalyzed oxidation of Menhaden oil as affected by emulsifiers. *J. Food Sci*. 63 (6), 997-1000

Doran Pauline M (1955) *Bioprocess Engineering Principles*. Academic Press, London (UK) 1995

Erickson MC (1998) Chemistry and function of phospholipids. In: *Food lipids*: *chemistry, nutrition and biotechnology.* Eds. C. C. Akoh, D. B. Min, Marcel Dekker, New York (USA) 283-332

Falch E, Storseth TR, Aursand A (2006) "Multi-component analysis of marine lipids in fish gonads with emphasis on phospholipids using high resolution NMR spectroscopy. *Chem. Phys. Lipids*. 144, 4-16

Faraji H, McClements DJ, Decker EA (2004) Role of continuous phase protein on the oxidative stability of fish oil- in-water emulsion. *J. Agric. Food Chem.* 52(14), 4558-4564

Frankel EN (2005) *Lipid oxidation.* Oily Press, Dundee (Scotland)

Fukuzawa K, Fujii T (1992) Peroxide dependent and independent lipid peroxidation: site-specific mechanism of initiation by chelated iron and inhibition by α-tocopherol. *Lipids* 27:227-233

Fukuzawa K, Seko T, Minami K, Terao J (1993) Dynamics of iron- ascorbate – induced lipid peroxidation in charged and uncharged phospholipid vesicles. *Lipids.* 28(6), 497-503

Fukuzawa K, Tadokoro T, Kishikawa K, Mukai K, Gebicki JM (1988) Site-specific induction of lipid peroxidation by iron in charged micelles. *Arch. Biochem. Biophys.* 260, 146-152

Garnier – Suillerot A, Tosi L, Paniago E (1984) Kinetic and mechanism of vesicle lipoperoxide decomposition by Fe (II). *Biochim. Biophys. Acta.* 794, 307-312

Genot C, Kansci G, Laroche M (1994) Measurement of phospholipid oxidation in model membranes by determination of oxygen consumption with a semi-automatic polarographic method. S*ciences des aliments.* 14, 673-682

Genot C, Metro B, Viau M, Bouchet B (1999) Characterisation and stability during storage of liposomes made of muscle phospholipids. *Lebensm. Wiss. U.- Technol.* 32, 167-174

Genot C, Meynier A, Riaublanc A (2003) Lipid oxidation in emulsions. In: *Lipid oxidation pathways* Ed. Kamal-Eldin A, AOCS Press. Champaign, IL, USA 190-244

Gibrat R, Grignon C (1982) Effect of pH on the surface charge density of plant membranes comparison of microsomes and liposomes. *Biochim. Biophys. Acta.* 692, 462-468

Girotti AW (1985) Mechanism of lipid peroxidation. *Free Radical Biol & Med.* 1985, 1, 87-95

Goddard JG, Sweeney GD (1987) Delayed, ferrous iron-dependent peroxidation of rat liver microsomes. *Arch Biochem Biophys.* 259(2), 372–381

Grung M, Svendsen YS, Liaaen-Jensen S (1993) The carotenoids of eggs of wild and farmed cod (*Gadus Morhua). Comp. Biochem. Physiol.* 106B (2), 237-242

Gutteridge JMC, Halliwell B (1990) The measurement and mechanism of lipid peroxidation in biological systems. *TIBS* 15, 129-135

Hu M, McClements DJ, Decker EA (2003) Impact of whey protein emulsifiers on the oxidative stability of salmon oil-in-water emulsions. *J. Agric. Food Chem.* 51(5), 1435-1439

Hunter RJ (1993) *Introduction to Modern Colloid Chemistry.* Oxford University Press, Oxford (UK) 1993

Hidalgo FJ, Nogales F, Zamora R (2005) Changes produced in the antioxidative activity of phospholipids as a consequence of their oxidation. *J. Agric. Food Chem.* 53, 659-662

Ingold KU (1962) Metal catalysis In: Symposium on Foods: Lipids and their Oxidation. Eds. Schultz HW, Day EA, Sinnhuber RO. Avi Publishing Co., Inc., Westport, Conn

Innis SM (1991) Essential fatty acids in growth and development. *Progress in Lipid research.* 30 (1), 39 -103

Ivanov II (1985) A relay model of lipid peroxidation in biological membranes. *J. Free Radicals Biol. Med.* 1, 247–253

Jacobsen C, Meyer AS, Adler-Nissen J (1999) Oxidation mechanism in real food emulsions: oil-water partition coefficients of selected volatile off-flavor compounds in mayonnaise. *Z. Lebensm Unters Forsch A* 208, 317-327

Jacobsen C, Hartvigsen K, Lund P, Adler-Nissen J, Holmer G, Meyer AS (2000a) Oxidation in fish-oil-enriched mayonnaise 2. Assessment of the efficacy of different tocopherol antioxidant systems by discriminant partial least squares regression analysis. *Eur. Food Res. Technol.* 210, 242-257

Jacobsen C, Hartvigsen K, Lund P, Thomsen MK, Skibsted LH, Adler-Nissen J, Holmer G, Meyer AS (2000b) Oxidation in fish-oil-enriched mayonnaise 3. Assessment of the influence of the emulsion structure on oxidation by discriminant partial least squares regression analysis. *Eur. Food Res. Technol.* 210, 242-257

Kates M (1991) *Techniques of lipidology isolation, analysis and identification of lipids.* 2nd revised edition, Elsevier Science Publishers (The Netherlands)

Kawakatsu M, Terao J, Matsushita S (1984) Phospholipid oxidation catalyzed by ferrous ion and ascorbic acid. *Agric. Biol. Chem.* 48(5) 1275-1279

Kellerby SS, Gu YS, McClements DJ, Decker EA (2006) Lipid oxidation in a menhaden oil-in-water emulsion stabilized by sodium caseinate cross-linked with transglutaminase. *J. Agric. Food Chem.* 54(26), 10222-10227

Klein RA (1970) The detection of oxidation in liposome preparations. *Biochim. Biophys. Acta* 210, 486-489

Kris-Etherton PM, Harris WS, Appel LJ (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* 106 (21), 2747-2757

Kulås E, Olsen E, Ackman RG (2003) Oxidation of fish lipids and its inhibition with tocopherols. In *Lipid oxidation pathways*. Ed. Kamal-Eldin A. AOCS Press Champaign, Illinois, USA

Kuzuya M, Yamada K, Hayashi T, Funaki C, Naito M, Asai K, Kuzuya F (1991) Oxidation of low-density lipoprotein by copper and iron in phosphate buffer. *Biochim Biophys Acta* 1084, 198-201

Labuza TP (1984) Application of chemical kinetics to deterioration of foods, *J. Chem. Educ.* 1984, 61 (4), 348-358

Labuza TP (1971) Kinetics of lipid oxidation in foods. *CRC Crit. Rev. food technol.*

Levenspiel O (1972) Chemical Reaction Engineering, 2nd ed, Wiley, New York

Løvaas E (2006) Marine phospholipids (MPL): resources, applications and markets. In: *Seafood research from fish to dish: quality, safety and processing of wild and farmed fish.* Ed. Luten, J. B., Jacobsen, C., Bekaert, K., Sæbø, A., Oehlenschlager, J., Wageningen Academic Publishers, The Netherlands 2006

Mabrouk AF, Dugan LR (1960) A kinetic study of the autoxidation of methyl linoleate and linoleic acid emulsions in the presence of sodium chloride. *J Am Oil Chem Soc.* **37**, 486-490

Makino K, Yamada T, Kimura M, Oka T, Ohshima H, Kondo T (1991) Temperature- and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. *Biophys Chem.* 41, 175-183

Makrides M, Hawkes JS, Neumann MA, Gibson RA (2002) Nutritional effect of including egg yolkin the weaning diet of breast – fed and formula fed infants: a randomized control trial. *Am. J. Clin. Nutr.* 75, 1084-1092

Mancuso JR, McClements DJ, Decker EA (1999) The effect of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. *J. Agric. Food Chem.* 47, 4112-4116

Mancuso JR, McClements DJ, Decker EA (2000) Iron-accelerated cumene hydroperoxide decomposition in hexadecane and trilaurine emulsions. *J. Agric. Food Chem.* 48, 213-219

McClements DJ (1999) *Food emulsions: principles, practise and techniques* CRC Press, Florida

Medina I, Sacchi R (1994) Acyl stereospecific analysis of tuna phospholipids via high resolution ^{13}C-NMR spectroscopy. *Chem. Phys. Lipids.* 70, 53-61

Mehansho H (2006) Iron fortification technology development: new approaches. *J. Nutr.* 136(4): 1059-63

McClements DJ, Decker EA (2000) Lipid oxidation in oil-in-water emulsions: impact of molecular environment on chemical reactions in heterogeneous food systems. *J Food Sci.* 65 (8), 1270-1282

Mehansho H (2006) Iron fortification technology development: New approaches. Journal of Nutrition 136(4), 1059-1063

Mei L, McClements J, Wu J, Decker EA (1998a) Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH, NaCl. *Food Chemistry.* 61(3), 307-312

Mei L, McClements J, Decker EA (1998b) Evidence of iron association with emulsion droplets and its impact on lipid oxidation. *J. Agric. Food Chem.* 46, 5072-5077

Min DB (1998) Lipid oxidation of edible oil. In: *Food lipids*: *chemistry, nutrition and biotechnology.* Eds. Akoh CC, Min DB, Mercel Dekker, New York (USA) 283-332

Minotti G, Aust SD (1989) The requirement for iron (III) in the initiation of lipid peroxidation by iron (II) and hydrogen peroxide. *J. Biol. Chem.* 262(3) 1098-1104

Minotti G, Aust SD (1987) The role of iron in the initiation of lipid peroxidation. *Chem. Phys. Lipids*, 262(3) 191-208

Monroig O, Navaro JC, Amat I, Gonzalez P, Amat F, Hontoria F (2003) Enrichment of *Artemia* nauplii in PUFA, phospholipids, and water-soluble nutrients using liposomes. *Aquaculture International* 2003, 11, 151-161

Nacka F, Cansell M, Meleard P Combe N (2001) Incorporation of α-tocopherol in marine lipid-based liposomes: *in vitro* and *in vivo* studies. *Lipids* 36(12), 1313-1320

Nawar WW (1996) Lipids. In: *Food chemistry.* Ed. Fennema OR, Marcel Dekker, NewYork (USA) 225-319

New RRC (1990) *Liposomes a practical approach.* IRL Press, Oxford

Nielsen NS, Timm-Heinrich M, Jacobsen C (2003) Comparison of wet-chemical methods for determination of lipid hydroperoxides. *J. Food Lipids.* 10, 35-50

Noguchi N, Yamashita H, Gotoh N, Yamamoto Y, Numano R, Niki E (1998) 2,2'-azobis (4- methoxy -2, 4 –dimethylvaleronitrile), a new lipid-soluble azo initiator: application to oxidations of lipids and low-density lipoprotein in solution and in aqueous dispersions. *Free Rad. Biol. Med.* 24, 259-268

Nuchi CD, McClements DJ, Decker EA (2001) Impact of tween 20 hydroperoxides and iron on the oxidation of methyl linoleate and salmon oil dispersions. *J. Agric. Food Chem.* 49(10), 4912-4916

Ohyashiki T, Kadoya A, Kushida K (2002) The role of Fe3+ on Fe2+ - dependent lipid peroxidation in phospholipid liposomes. *Chem. Pharm. Bull.* 50(2), 203-207

O'Keefe SF (1998) Nomenclature and classification of lipids. In: *Food lipids*: *chemistry, nutrition and biotechnology.* Eds. Akoh CC, Min DB. Mercel Dekker, New York (USA) 283-332

Osinchak JE, Hultin HO, Zajicek OT, Kelleher SD, Huang CH (1992) Effect of NaCl on catalysis of lipid oxidation by the soluble fraction of fish muscle. *Free Radical Biol Med.* 12, 35-41

Pearson AM, Gray JI, Wolzak AM, Horenstein NA (1983) Safety implications of oxidized lipids in muscle foods. *Food Technol.* 37(7), 121-129

Pearson AM, Love JD, Shorland FB (1977) "Warmed – over" flavour in meat, poultry and fish. In: *Advances in Food Research* Ed by Chrichester C. O., Merck, E. M. and Steward, G. F. Academic Press, New York, (USA), 1-74

Peet M, Stokes C (2005) Omega-3 fatty acids in the treatment of psychiatric disorders. *Drugs* 65(8), 1051-1059

Phleger CF, Nelson MM, Mooney BD, Nichols PD (2002) Interannual and between species comparison of the lipids, fatty acids and sterols of Antarctic krill from the US AMLR Elephant Island survey area. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*. 131(4), 733-47

Pokorny J (1987) Major factor affecting the autoxidation of lipids. In Chan HWS (ed) Autoxidation of unsaturated lipids. Academy Press, London

Pond D, Watkins J, Proddle J, Sargent J (1995) Variation in the lipid content and composition of Antartic krill Euphausia superba at Sourth Georgia. *Mar. Ecol. Prog. Ser.* 117, 49-57

Reische DW, Lilliard DA, Eitenmiller RR (1998) Antioxidants. In: *Food lipids: chemistry, nutrition and biotechnology.* Eds. Akoh CC, Min DB. Mercel Dekker, New York, (USA) 423-448

Sato T, Sunamoto J (192) Recent aspects in the use of liposomes in biotechnology and medicine. *Prog. Lipid Res.* 31, 345-372.

Schaich KM (1992) Metals and lipid oxidation. Contemporary issues. *Lipids.* 27(3), 209-218

Schiller J, Sub R, Arnhold J, Fuchs B, Lebig J, Muller M, Petkovic M, Spalteholz H, Zschornig O, Arnold K (2004) Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectroscopy in lipid and phospholipids research. *Prog. Lipid Res.* 43, 449-488

Shahidi F, Wanasundara UN (1998) Methods of measuring oxidative rancidity in fats and oils. In: *Food lipids*: *chemistry, nutrition and biotechnology.* Eds. C. C. Akoh, D. B. Min, Mercel Dekker, New York (USA), 283-332

Shimada K, Okada H, Matsuo K, Yoshioka S (1996) Involvement of chelating action and viscosity in the antioxidative effect of xanthan in an oil/water emulsion. *Biosci. Biotech. Biochem*. 60(1), 125-127

Silvestre MPC, Chaiyasit W, Brannan RG, McClements DJ, Decker EA (2000) Ability of surfactant headgroup size to alter lipid and antioxidant oxidation in oil-in-water emulsions. *J. Agric. Food Chem.* 48(6), 2057-2061

Subczynski WK, Hyde JS, Kusumi A (1989) oxygen permeability of phosphatidylcholine - cholesterol membranes. *Biophysics.* 86, 4474-4478

Tadolini B, Hakim G (1996) The mechanism of iron (III) stimulation of lipid peroxidation. *Free Rad Res.* 25(3), 221-227

Tadolini B, Gabrini L, Menna C, Pinna GG, HakimG (1997) Iron (III) stimulation of lipid hydroperoxide dependent lipid peroxidation. *Free Rad. Res.* 27:563-576

Tan CP, Che Man YB, Selamat J, Yusoff MSA (2001) Application of Arrhenius kinetics to evaluate oxidative stability in vegetable oils by isothermal differential scanning calorimetry. *J. Am. Oil Chem. Soc.* 78(11), 1133-1138

Tang L, Zhang Y, Qian Z, Shen X (2000) The mechanism of Fe2+ - initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical. *Biochem. J.* 352, 27-36

Terao J (1989) Stabilization of phosphatidylethanolamine hydroperoxides in human plasma. *Biochim. Biophys. Acta* 1003, 221-224

Tocanne JF, Teissie J (1990) Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion of protons in membrane model systems. *Biochim. Biophys. Acta.* 111-142

Tocher DR, Sargent JR (1984) Analyses of lipids and fatty acids in ripe roes of some Northwest European marine fish. *Lipids.* 19(7), 492-499

Tolasa S, Cakli S, Ostermeyer U (2005) Determination of astaxanthin and canthaxanthin in salmonid. *Eur Food Res Technol.* 221, 787-791

Tong LM, Sasaki S, McClements DJ, Decker EA (2000) Antioxidant activity of whey in a salmon oil emulsion. *J Food Sci.* 65 (8), 1325-1329

Vercellotti J*R*, St. Angelo AJ, Spanier AM (1992) Lipid oxidation in foods: An overview. In: *Lipid Oxidation in Food.* American Chemical Society, Washington, D.C. 1-11

Wang JY, Wang ZY, Kouya T, Shibata T, Ueki T (1994) Significance of amino groups of phosphatidylethanolamine in phospholipid peroxidation of mixed liposomes. *Chem. Phys. Lipids.* 71, 197-203

Welch KD, Davis TZ, Aust SD (2002) Iron autoxidation and free radical generation: effects of buffer, ligands and chelators. *Archiv Biochem Biophys.* **397**(2), 360-369

Yamamoto Y, Niki E, Kamiya Y, Shimasaki H (1984) Oxidation of phosphatidylcholines in homogeneous solution and water dispersion. *Biochim. Biophys. Acta.* 795, 332-340

Yamamoto Y, Niki E (1988) Interaction of α-tocopherol with iron: antioxidant and prooxidant effects of α-tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron. *Biochim. Biophys. Acta.* 958, 19-23

Yoshida Y, Niki E (1992) Oxidation of methyl linoleate in aqueous dispersions induced by copper and iron. *Arch. Biochem. Biophys.* 295 (1), 107-114

Yin D, Lingnert H, Ekstrand B, Bruk UT (1992) Fenton reagents may not initiate lipid peroxidation in an emulsified linoleic acid model system. *Free Radical Biol & Med.* 13, 543-556

Zimmermann M (2004) The potential of encapsulated iron compounds in food fortification: a review. *Int. J. Vitam. Nutr. Res.* 74(6), 453-461

Paper I

Paper I is not included due to copyright.

Paper II

The role of iron in peroxidation of PUFAs in liposomes

Revilija Mozuraityte[1], Turid Rustad[1] and Ivar Storrø[2]

[1]- Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway

[2]-SINTEF Fisheries and Aquaculture, Trondheim, Norway

Correspondence: Turid Rustad, Department of Biotechnology, Norwegian University of Science and Technology, 7491 Trondheim, Norway. Phone: +47 73 59 40 66, Fax: +47 73 59 33 37; e-mail: turid.rustad@biotech.ntnu.no

Abbreviations:

CumOOH - cumene hydroperoxide, DPPD - N, N'-Diphenyl-p-phenylene-diamine, PUFA polyunsaturated fatty acid, EDTA - ethylenediaminetetraacetic acid, LOOH lipid peroxide, L$^{\cdot}$ - lipid radical, LOO$^{\cdot}$ - peroxy radical, LO$^{\cdot}$ - alkoxy radical, MES - 2-morpholinethanoesulfonic acid, PL-phospholipids, OUR – oxygen uptake rate, PV peroxide value, TBARS thiobarbituric reactive substances, TPP – triphenylphosphine.

Abstract

This work discusses iron catalysed lipid oxidation in marine phospholipid liposomes. Oxygen consumption was used as a method to study lipid oxidation at pH 5.5 and 30°C. The relationship between consumed oxygen and amount of peroxides (PV) and thiobarbituric reactive substances (TBARS) formed, indicated that both Fe^{2+} and Fe^{3+} catalysed lipid oxidation.When Fe^{2+} was added to liposomes at a concentration of approx. 10 µM, an initial drop in dissolved oxygen (oxygen uptake rate >258µM/min), followed by a slower linear oxygen uptake (oxygen uptake rate 4-6µM/min), was observed. Addition of Fe^{3+} induced only the linear oxygen uptake. The initial fast drop in dissolved oxygen was due to oxidation of Fe^{2+} to Fe^{3+} by lipid peroxides (rate of 79 µM Fe^{2+}/min). No oxidation of Fe^{2+} was observed in oxygen saturated buffer at pH 5.5, proving that Fe^{2+} was oxidized by the added lipids. Fe^{3+} is reduced by peroxides to Fe^{2+} at a slow rate (0.25µM of Fe^{3+}/min at 30^{o}C) in a pseudo-first order reaction. When equilibrium between Fe^{2+} and Fe^{3+} was achieved, the linear oxygen uptake was observed and Fe^{3+} became the rate limiting factor in the circulation between Fe^{3+} and Fe^{2+}. Both alkoxy and peroxy radicals are presumably formed by breakdown of peroxides by Fe^{2+} and Fe^{3+}. These radicals react with fatty acids giving a lipid radical reacting with oxygen.

1 Introduction

Heavy metals, particularly those with two or more valency states, increase the rate of lipid oxidation. The mechanism of iron-catalysed lipid peroxidation is proposed to depend on the presence or absence of preformed lipid hydroperoxide (LOOH) and is divided into LOOH-independent (or iron dependent) and LOOH-dependent initiation [1]. LOOH - independent oxidation occurs in lipid without peroxides. The mechanism of initiation of LOOH – independent oxidation is still not clear. However, even with a low concentration of peroxides, the peroxide dependent oxidation reactions may become dominant. As almost all lipids contain at least traces of peroxides, studies of lipid oxidation in aqueous colloidal systems suggest that during the propagation step, the interaction between lipid hydroperoxides located at/or near the droplet surface, and transition metals, originating in the aqueous phase, is the most common cause of oxidative instability [2-5]. In several studies [3, 6] it was observed that when peroxides were removed, iron did not induce lipid peroxidation.

LOOH dependent initiation has been proposed to occur through two pathways [2]:

a) LOOH breakdown by Fe^{3+} and subsequent hydrogen abstraction by $LOO^{\cdot}$ (reaction 1 and 2);

$$LOOH + Fe^{3+} \rightarrow LOO^{\cdot} + H^{+} + Fe^{2+} \quad (1)$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad (2)$$

or

b) Fe^{2+} accelerates lipid oxidation by breaking down lipid peroxides to free radicals (reaction 3 and 4)

$$LOOH + Fe^{2+} \rightarrow LO^{\cdot} + OH^{-} + Fe^{3+} \quad (3)$$

$$LO^{\cdot} + LH \rightarrow LOH + L^{\cdot} \quad (4)$$

Lipid radical ($L^{\cdot}$) can react with diradical triplet oxygen (O_2) to form peroxy radical ($LOO^{\cdot}$) [7].

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad (5)$$

The interaction of $L^{\cdot}$ with O_2 has a very low activation energy and the reaction rate is very fast compared to other radical reactions in lipid oxidation [7]. The $LOO^{\cdot}$ formed can react further in reaction 2 and form lipid radical ($L^{\cdot}$). Hence a single formed $L^{\cdot}$ can result in conversion of several

fatty acid side chains into lipid hydroperoxides, accompanied by oxygen consumption. The decomposition of peroxy radicals ($LOO^{\cdot}$) also results in the accumulation of short-chain lipid peroxidation end products, mostly aldehydes [8].

A possible circulation between Fe^{2+} and Fe^{3+} through peroxide decomposition reactions 1 and 3 was proposed by Pokorny [9] to be an important source of free radicals, but this theory does not seem to have attracted much attention.

Fe^{2+} is reported to decompose peroxides at a higher rate than Fe^{3+} ions [10]. This could be attributed to a lower activation energy for the cleavage of O-O bond (184 kJ/mol) than that for the O-H bond (377 kJ/mol) [7]. When oxygen is abundant, termination reactions between alkoxy ($LO^{\cdot}$) or peroxy ($LOO^{\cdot}$) radicals take place to form non radical oxidation products [11]. When the oxygen concentration is low, termination reactions between $L^{\cdot}$ and $LOO^{\cdot}$, $LO^{\cdot}$ or $L^{\cdot}$ occur. These termination reactions prevent further autoxidation reactions [11].

Tang et al. [12] also proposed that reaction between Fe^{2+} and lipid peroxide was responsible for the initiation of lipid peroxidation, but lipid peroxidation could only start when the concentration of Fe^{2+} was reduced to a critical value. Before the critical Fe^{2+} concentration was reached, a latent period was observed. During this period, the concentration of Fe^{2+} was reduced to a level where the peroxy and alkoxy radicals could no longer be converted to non-radical species by their reaction with the remaining Fe^{2+} (6, 7) and their attack on the fatty acid chain became dominant (2, 4).

$$Fe^{2+} + LOO^{\bullet} + H^{+} \rightarrow LOOH + Fe^{3+} \qquad (6)$$

$$Fe^{2+} + LO^{\bullet} + H^{+} \rightarrow LOH + Fe^{3+} \qquad (7)$$

Fukuzawa et al. [3] found that Fe^{2+} ions alone did not promote lipid oxidation, but a high concentration of Fe^{2+} ions induced a slight peroxidation. Adding ascorbic acid together with Fe^{2+} ions promoted lipid oxidation and the increase in oxidation was explained by the reduction of Fe^{3+} to Fe^{2+} by ascorbic acid and thereby the ability to significantly promote lipid oxidation in liposomes.

When oxygen uptake was used to study lipid oxidation in liposomes [13,14], a fast decrease in concentration of dissolved oxygen was observed for a short period after Fe^{2+} addition, which was followed by a slower linear decrease proportional to the added Fe^{2+} concentration [13]. In oxidation studies of phospholipid emulsions by Fe^{2+} and Fe^{3+} ions, Kawakatsu et al. [15] observed a similar type of changes during formation of conjugated dienes. Genot et al. [16] also observed a faster drop followed by a slower decrease in dissolved oxygen after addition of Fe^{2+} - ascorbate mixture to

liposomes. Chaiyasit et al. [17] observed a rapid decomposition of cumene hydroperoxide after addition of Fe^{2+} in emulsions prepared with hexadecane (nonoxidisable lipid that does not form additional peroxides in the presence of radicals) within the first 0.2h. After 0.2h only small changes in peroxide value was observed. This was explained by depletion of Fe^{2+} with the resulting Fe^{3+} being unable to significantly promote peroxide decomposition. Fukuzawa et al. [6] explained the oxidation induced by Fe^{2+} to be initiated by peroxide breakdown to alkoxy radical which was accompanied by oxygen consumption. The rapidly reached constant rate was explained by the limiting availability of LOOH.

The objective of this work has been to study the role of iron in peroxidation of phospholipids in liposomes. The oxidation pathways during iron induced oxidation of unsaturated fatty acids are discussed.

2 Materials and methods

Chemicals. 2-morpholinethanoesulfonic acid (MES), 2,2–dipyridyl, triphenylphosphine (TPP), $FeSO_4$, $FeCl_3$, butylated hydroxytoluene, thiobarbituric acid, 1,1,3,3-tetraethoxypropane (TEPE) was from Sigma-Aldrich. N, N'-Diphenyl-p-phenylene-diamine (DPPD) and cumene hydroperoxide (CumOOH) was from Fluka, ethylenediaminetetraacetic acid (EDTA) and $(NH_4)_2Fe(SO_4)_2$ (titrisol) from Merck.

Phospholipids. Phospholipids used in the experiments were isolated from North Atlantic cod (*Gadus morhua*) roe. Before extraction, the cod roe was kept at –40°C.

The extraction of total lipids was performed according to the method of Bligh and Dyer [18]. Phospholipids were isolated from total lipids using the acetone precipitation method [19], with a few modifications as described by Mozuraityte et al. [13].

Characterisation of the phospholipids used is given in Mozuraityte et al. [14].

Preparation of liposomes. Liposomes were made as described by Mozuraityte et al. [13]. Chloroform solution of phospholipids was evaporated to dryness with a stream of nitrogen gas. The residual solvent was completely evaporated under vacuum. The liposomes which were prepared with N, N'-Diphenyl-p-phenylene-diamine (DPPD) or triphenylphosphine (TPP) were prepared from phospholipids that had been mixed with either TPP or DPPD in chloroform and then dried under nitrogen. Phospholipids were sonicated in a 5mM MES buffer pH 5.5 with an MSE Ultrasonic Disintegrator Mk2 (MSE Scientific instruments, Sussex, England), at a lipid concentration of 30mg/ml. The lipid concentration in the liposome solution was 6mg/ml in all

experiments if nothing else is mentioned. To verify the experiment temperature, the temperature was measured directly in the cell. All experiments were performed at 30°C.

Oxidation experiments. The consumption of dissolved oxygen by liposomes in a closed, stirred, water jacketed cell, was used as a measure of lipid oxidation. The concentration of dissolved oxygen was measured continuously by a polarographic oxygen electrode (Hansatech Instrument Ltd., Norfolk, UK). When measuring dissolved oxygen concentration, background oxygen uptake rate was observed for 4-6 min before Fe^{2+} or Fe^{3+} injection. 20µl of 0.375 or 0.75 mM $FeSO_4$ or $FeCl_3$ working solution, obtaining a final concentration of 7.5 or 15 µM in the liposome solution respectively was injected through a capillary opening in the cell to catalyse lipid oxidation. A stock solution of 15mM $FeSO_4 \cdot 7H_2O$ (99.5%, Merck) or $FeCl_3$ in 0.5 N HCl was prepared fresh every month. The working solution (0.375mM Fe^{2+} or Fe^{3+} in 5mM MES-buffer, pH 5.5) was prepared daily.

For studies of the relationship between oxygen uptake, PV and TBARS, the experiments were performed in closed test tube with screw cap and rubber septum. The vessel, with a glass ball inside to facilitate mixing, was completely filled with liposome solution and kept in a water bath at 30°C. To catalyse the lipid oxidation, Fe^{2+} or Fe^{3+} working solution with final concentration 7.5 µM for Fe^{2+} and 15 µM for Fe^{3+} in the liposome solution was injected. After the desired reaction time, the oxidation was stopped by the injection of a solution of EDTA (0.75mM in 5mM pH 5.5 MES buffer) with a final concentration of 15 µM. After oxidation, the concentration of dissolved oxygen in the liposome solution was measured. 7ml of the liposome solution was used for fat extraction, and subsequent PV measurements. Chloroform/methanol (1:1) was used for the extraction of lipids from liposomes. Peroxide value (PV) was analysed by the Ferric thiocyanate method as described by the International Dairy Federation [20], and modified by Ueda et al. [21] and Undeland et al. [22]. Cumene hydroperoxide was used as standard for quantitative peroxide analyses. The analysis was performed in triplicate.

Thiobarbituric acid reactive substances (TBARS) were analysed in the water phase as described by McDonald and Hultin [23], using 0.2ml of liposome solution, 0.8ml H_2O, and 2ml TBAR reagent. The absorbance values were compared to a standard curve prepared with 1,1,3,3-tetraethoxypropane for the calculation of TBARS concentrations. The analysis was performed in triplicate.

Determination of the Fe^{2+} oxidation. Fe^{2+} concentration measurements were performed as described by Fukuzawa and Fujii [24]. 40µl of 375mM 2,2–dipyridyl (final concentration 5mM)

was added to 3 ml of liposome solution and the absorbance was measured at 520nm. Fe^{2+} concentration was calculated using extinction coefficient 9600 $cm^{-1}M^{-1}$. The extinction coefficient was obtained from a standard curve based on $(NH_4)_2Fe(SO_4)_2$ (titrisol) in MES buffer.

Determination of Fe^{2+} production rate during reduction of Fe^{3+} was performed by following the increase in absorbance due to production of 2,2-dipyridyl-Fe^{2+} complex as a function of time. 2, 2-dipyridyl solution was added to the samples after different oxidation times, mixed with 2,2–dipyridyl, quickly transferred to the cuvette, and the increase in absorbance was recorded. The concentration of Fe^{2+} at the time of withdrawal of the sample was found by extrapolation back to zero time. The rate of Fe^{3+} reduction was calculated from the increase in the absorbance of the 2,2-dipyridyl-Fe^{2+} complex .

Microsoft Excel was used for data processing and statistical analyses. The significance level was set at 95% (p=0.05).

3 Results and discussion

Oxidation catalysed by Fe^{2+} and Fe^{3+}

Oxidation of polyunsaturated fatty acids (PUFA's) catalysed by either Fe^{2+} or Fe^{3+} was performed in a closed system containing solution of liposomes. Both Fe^{2+} and Fe^{3+} induced lipid oxidation of cod roe phospholipids in the liposomes. The oxidation was measured as consumption of dissolved oxygen accompanied by an increase in peroxides (PV) and thiobarbituric acid reactive substances (TBARS) after addition of iron (Fig. 1). Data from the Fe^{2+} and Fe^{3+} induced lipid oxidation experiments was pooled together and a significant linear relationship ($p<0.05$) between consumed oxygen and amount of PV and TBARS was observed indicating that both Fe^{2+} and Fe^{3+} ions catalysed the same type of oxidation. Low concentrations of Fe^{2+} or Fe^{3+} (i. e. 7.5 μM) resulted in consumption of all dissolved oxygen (at 30°C ~230μM) and formation of ~100μM of PV (calculated with cumene hydroperoxide as a reference) and ~20 μM of TBARS, indicating that a single iron molecule leads to formation of several molecules of peroxides and TBARS (Fig. 1).

Initial drop in dissolved oxygen

When Fe^{2+} was added to liposomes to catalyse lipid oxidation, an *initial drop* in concentration of dissolved oxygen was observed, this was followed by a slower *linear decrease* in concentration of dissolved oxygen (Fig. 2). The initial drop in dissolved oxygen was proportional to the added Fe^{2+} concentration ($p<0.05$) (Fig. 3). The amount of oxygen consumed during the initial drop was observed to be 5.6 ± 0.5 times higher than the amount of added iron (Fig. 3). As a linear

relationship between consumed oxygen vs peroxide formation was observed (Fig. 1), this indicates that also during the initial drop, caused by Fe^{2+}, a single Fe^{2+} ion led to consumption of several oxygen molecules and generation of several peroxides.

Repetitive addition of Fe^{2+} to the same liposome solution induced repetitive initial oxygen drops proportional to the added Fe^{2+} concentration (7.5μM), which shows that the initial drop in oxygen concentration is an equilibrium reaction involving Fe^{2+}. However, when Fe^{3+} was added to the liposome solution, the initial drop in dissolved oxygen concentration was hardly detectable (Fig. 2). To measure the rate of the initial drop in dissolved oxygen, the concentration of phospholipids in the liposome solution was reduced. The rate of oxygen consumption during the initial drop was clearly dependent on the phospholipid concentration in liposome solution (Fig. 4a) being slower at lower lipid concentration.

After addition of Fe^{2+} to liposomes, a fast decrease in Fe^{2+} concentration during the initial oxygen uptake phase was observed (Fig. 4b) which was due to oxidation of Fe^{2+} to Fe^{3+}. No changes in Fe^{2+} concentration was observed in buffer only, which proves that oxygen itself did not oxidise Fe^{2+} at pH ~5.5. Reduced Fe^{2+} oxidation rate was observed in liposome solutions with low phospholipid concentration, indicating that phospholipids were the rate limiting factor at the low lipid concentrations used. Peroxides are the proposed substrates for initiation of iron induced lipid oxidation according to the peroxide-dependent oxidation mechanism [2-5]. At the initiation of oxidation, in a liposome solution with a phospholipid concentration of 6mg/ml, the peroxide concentration was o 36.2±4.1 μM. When the lipid concentration in the liposome solution was lowered (from 6 to 0.05 mg/ml), the concentration of peroxide (substrate for reaction 1) and/or allylic center of unsaturated fatty acids (substrate for reactions 3 and 4) available per Fe^{2+} unit probably became limiting, resulting in a lower oxidation rate of phospholipids and Fe^{2+}.

Triphenylphosphine (TPP) breaks down peroxides to alcohols [3] and was added to the phospholipids prior to the formation of liposomes to reduce the peroxide value. When different concentrations of TPP (0, 100, 200 and 400μM) were added to the phospholipids, the peroxide concentration in the liposome solution containing 6mg/ml phospholipids, was reduced (36.2, 29.8, 23.6 and 22.8 μM respectively). To remove peroxides, a surplus of 400μM of TPP was used. However, peroxides were still measured even in the liposomes made from phospholipids with the highest TPP concentration. Partial reduction of peroxides by an added surplus of TPP was also found by Ohyashiki et al. [25]. The partial reduction of the PV values could be explained by selective decomposition of certain types of PL-peroxides by TPP. Another explanation could be that some peroxides were produced during liposome preparation, or during phospholipid extraction for PV measurement. Inhibition of oxygen uptake was observed in liposomes treated with TPP (Fig. 5).

No initial oxygen consumption was observed at concentration of 200 – 400 μM TPP. At the same concentrations of TPP the linear oxygen uptake was also completely inhibited. These results suggest that lipid peroxides are necessary to initiate lipid peroxidation by Fe^{2+}, which is in agreement with the findings of Fukuzawa et al [3] and Tadolini et al. [26].

Constant oxygen uptake rate

During the initial drop in dissolved oxygen, Fe^{2+} was oxidised to Fe^{3+}. When Fe^{2+} concentration reached constant level (~1μM), a constant oxygen uptake was observed (Fig. 4 a, b). When EDTA was added at double the concentration of Fe^{2+}, the constant oxygen uptake was immediately stopped (results not shown). This indicates that iron ions were involved in oxidation reactions and continuously producing radicals resulting in oxidation, measured as constant oxygen uptake

Fe^{2+} concentration measurements are based on the rapid reaction of 2,2-dipyridyl with Fe^{2+} forming a pink coloured complex within seconds (Fig. 6a). Absorbance measurements of this pink coloured complex were used to study the changes in Fe^{2+} concentration during oxidation. When Fe^{2+} was added to liposomes already containing 2,2-dipyridyl, only the added concentration of Fe^{2+} was measured (Fig. 5b, oxidation time 0s). This indicates that 2,2-dipyridyl complexes Fe^{2+}, preventing oxidation of Fe^{2+} to Fe^{3+} (Fig. 6b). When 2,2-dipyridyl was added to the liposomes after different periods of oxidation induced by Fe^{2+}, the absorbance increased with time until the Fe^{2+} concentration reached the initial added concentration of Fe^{2+} (Fig. 6b). This shows that the increase in absorbance was due to continuous formation of 2,2-dipyridyl - Fe^{2+} complex, at the same rate as formation of Fe^{2+} from Fe^{3+}. The increase in absorbance during the first 20 min after addition of 2,2–dipyridyl to the liposome solution, was used to calculate the rate of reduction of Fe^{3+} to Fe^{2+}. Initial rate of reduction of Fe^{3+} to Fe^{2+} varied from 0.15-0.22 μM/min at room temperature and ~0.25 μM/min at 30^{o}C (Table 2) and was dependent on Fe^{3+} concentration following a pseudo first order reaction. The rate constant was determined to 0.015±0.001 min^{-1} at 20°C and 0.020±0.001 min^{-1} at 30°C. The low activation energy for this process indicates that it might be a transport limited process [27].

The formation of Fe^{2+} from Fe^{3+} most probably occurred due to reaction 1 producing peroxy radicals. The formed Fe^{2+} further reacts with peroxides producing alkoxy radicals. This leads to a redox cycling between Fe^{2+} and Fe^{3+}, where the reduction of Fe^{3+} is a limiting factor. When the equilibrium is established between Fe^{2+} and Fe^{3+} the slower constant oxygen uptake is observed.

At equilibrium the peroxide decomposition by Fe^{2+} (reaction 3) and Fe^{3+} (reaction 1) could be described by the following equation:

$$[Fe^{2+}]*[LOOH]*k1=[Fe^{3+}]*[LOOH]*k2$$

where: $[Fe^{2+}]$, $[[Fe^{3+}]$ is the iron concentration, [LOOH] - peroxide concentration and k1, k2 are reaction constants for reaction 3 and 1 respectively.

At equilibrium, the concentration of Fe^{2+} was found to be less than 1/10 of the Fe^{3+} concentration. The ratio for the rate constants for this reaction was calculated to be $k2/k1 \approx 1/10$ as the peroxide concentration is the same for both reactions.

DPPD and lipid oxidation

The radical chain reaction is supposed to involve peroxy radicals $LOO^{\bullet}$. Addition of the peroxy radical scavenger, N, N'-diphenyl-p-phenylene-diamine (DPPD) [28], should therefore stop the autooxidation reactions. Addition of DPPD to the liposomes reduced both Fe^{2+} and Fe^{3+} induced oxygen uptake (Fig. 8), which supports the participation of peroxy radicals in the oxidation of unsaturated fatty acids.

Lipid oxidation in liposomes

From the observations described above we propose that Fe^{2+} induced a rapid oxidation in liposomes, which was observed as an initial drop in dissolved oxygen. At 6mg/ml lipid concentration and 15 μM Fe^{2+} the rate was >258 μM O_2/min. As Fe^{2+} was oxidised to Fe^{3+}, the rate of oxidation reached a constant rate of 4-6 μM oxygen/min proportional to the added iron concentration [13]. A decrease in oxidation rate when Fe^{2+} was oxidised to Fe^{3+} was also observed by Yoshida and Niki [29], but in their studies the decrease was not quantified.

When Fe^{2+} was added to the solution of liposomes, the initial oxygen uptake was due to radical production via breakdown of peroxides by Fe^{2+} (Fig. 7). As oxidation rate of Fe^{2+} via peroxide breakdown reaction was observed to be 79μM/min, the alkoxy radical formation rate was also supposed to be 79μM/min. One alkoxy radical further propagated lipid peroxidation by chain branching leading to production of several peroxides accompanied by oxygen consumption (lower cycle in Fig. 7) as a rate of 258 μM O_2/min was measured during the initial drop in oxygen. When

equilibrium between Fe^{2+} and Fe^{3+} was achieved after the initial fast oxygen uptake, a constant rate of oxygen uptake was observed.

Fe^{3+} also induced oxidation of lipids as PV and TBARS values increased together with oxygen consumption (Fig. 1). No initial drop in dissolved oxygen was observed after addition of Fe^{3+} to the liposome solution, only the linear oxygen uptake was observed. Reaction of Fe^{3+} with peroxides was slower (~0.25 μM Fe^{3+}/min) than the oxidation of Fe^{2+} by peroxides measured during the initial drop. Due to this, reduction of Fe^{3+} became the limiting factor for circulation between Fe^{2+} and Fe^{3+} in the *radical generating cycle* (Fig. 7 upper cycle) when equilibrium was reached. The rate of radical production in the *radical generating cycle* can be calculated to 0.5 μM/min based on equal oxidation and reduction rates of the iron species. Both peroxy and alkoxy radicals formed in the radical production cycle are proposed to subtract bisallylic hydrogen from fatty acids [10]. The generated lipid radical (L·) take part in the *peroxide production cycle* leading to formation of several peroxides as shown in the lower cycle in Fig. 7. The oxygen consumption at constant oxygen uptake was observed to be 4-6 μM O_2/min. Constant oxygen uptake rate has been shown to be independent of the concentration of dissolved oxygen from 100 % saturation down to at least 5% [13]. To achieve constant oxygen uptake rate the concentration of radicals, in specific lipid radicals (L·), must be constant. Their concentrations are regulated by the production in the radical generating cycle and removal by the termination reactions. As the radical concentrations are constant when the oxidation rate is constant, the production rate and the termination rate should be equal. Based on these assumptions it is possible to calculate that the peroxy- and lipid radicals in average would react approx. 10 times in the peroxide producing cycle before they are removed by termination reactions.

According to our proposed theory, the linear uptake of oxygen when adding Fe^{2+} or Fe^{3+} should be the same. Repetitive addition of Fe^{2+} and Fe^{3+} were used to catalyse oxidation in liposomes (Figure 2). After the first addition of iron, the rate of linear oxygen uptake (OUR) per mole of iron was similar both for Fe^{2+} and Fe^{3+} (Table 3). However, the second addition gave a slightly lower OUR per mole of Fe^{3+} than for Fe^{2+}. To explain this observation Fe^{2+} and Fe^{3+} working solutions were made at pH 2 and 3.5 and oxidation experiments performed with 15μM iron. The oxidation rates were lower when iron solutions with higher pH were used. The increase in working solution pH led to approximately 47% reduced OUR for Fe^{2+} and 90% for Fe^{3+} which reflects the solubility product constants, $5*10^{-17}$ and $3*10^{-39}$ for the corresponding hydroxides [30]. This may indicate that iron hydroxides [31] play a role in determining the concentration of active iron on the surface of the liposomes. Repetitive additions of Fe^{3+} probably increased the fraction of

iron hydroxide upon addition into the measuring cell more than addition of Fe^{2+}, leading to lower oxidation rates.

After addition of Fe^{2+} to liposomes an increase in PV and TBARS values followed the oxygen consumption (Fig. 9). When all the dissolved oxygen was depleted, the decomposition of peroxides by iron could occur through the radical production cycle, with the consumption of peroxides. Due to this reaction a decrease in PV values was observed after depletion of dissolved oxygen in the liposome solution.

Conclusions

Both Fe^{2+} and Fe^{3+} catalysed lipid oxidation. When Fe^{2+} was used to induce oxidation of phospholipids in liposomes, a fast initial drop in dissolved oxygen, followed by a slower linear oxygen uptake was observed. Fe^{3+} only induced the linear oxygen uptake. The initial drop in dissolved oxygen, after adding Fe^{2+} to liposomes, was due to a conversion of Fe^{2+} to Fe^{3+}. Fe^{2+} was oxidised to Fe^{3+} (rate >79 µM Fe^{2+}/min at 0.6% lipid concentration and 15µM of Fe^{2+} added) and alkoxy radicals were formed. No oxidation of Fe^{2+} was observed in oxygen saturated buffer at pH 5.5, which proved that Fe^{2+} was oxidized by the added lipids. The Fe^{3+} formed break down peroxides generating peroxy radicals but at a slower rate, becoming a limiting factor in the circulation between Fe^{2+} and Fe^{3+}. Fe^{3+} is reduced by peroxides to Fe^{2+} at a slow rate (0.25µM of Fe^{3+}/min at 30^{o}C) in a pseudo-first order reaction with respect to the Fe^{3+} concentration. When equilibrium between Fe^{2+} and Fe^{3+} was achieved, the linear oxygen uptake was observed. Both alkoxy and peroxy radicals formed from breakdown of peroxides by Fe^{2+} and Fe^{3+} reacted with fatty acids giving a lipid radical capable of reacting with oxygen. The net result is production of lipid peroxides accompanied by oxygen consumption.

Acknowledgement

This work was performed within the Integrated Research Project SEAFOODplus, contract No FOOD-CT-2004-506359. The partial financing of the work by the European Union is gratefully acknowledged.

References

1. Girotti AW (1985) Mechanism of lipid peroxidation. Free Radical Biol Med 1:87-95
2. Frankel EN (1998) Lipid oxidation. Lipid oxidation. Oily Press, Dundee

3. Fukuzawa K, Seko T, Minami K, Terao J (1993) Dynamics of iron-ascorbate – induced lipid peroxidation in charged and uncharged phospholipid vesicles. Lipids 28: 497-503
4. Nawar WW (1996) Lipids. In: Fennema OR (ed), Food chemistry. Marcel Dekker, NewYork (USA)
5. Mei L, McClements J, Wu J, Decker EA (1998a) Iron-catalyzed lipid oxidation in emulsion as affected by surfactant, pH, NaCl. Food Chem 61:307-312
6. Fukuzawa K, Tadokoro T, Kishikawa K, Mukai K, Gebicki JM (1988) Site – specific induction of lipid peroxidation by iron in charged micelles. Arch Biochem Biophys 260:146-152
7. Min DB (1988) Lipid oxidation of edible oil. In: Akoh CC, Min DB (eds) Food lipids: chemistry, nutrition and biotechnology. Marcel Dekker, New York (USA)
8. Minotti G, Aust S (1989) The role of iron in oxygen radical mediated lipid peroxidation. Chem Biol Interactions 71:1-19
9. Pokorny J (1987) Major factor affecting the autoxidation of lipids. In Chan HWS (ed) Autoxidation of unsaturated lipids. Academy Press, London
10. Frankel EN (2005) Lipid oxidation. Oily Press, Dundee
11. Colakoglu A (2007) Oxidation kinetics of soybean oil in the presence of monoolein, stearic acid and iron. Food Chem 101: 724-728
12. Tang L, Zhang Y, Qian Z, Shen X (2000) The mechanism of Fe2+ - initiated lipid peroxidation in liposomes: the dual function of ferrous ions, the roles of the pre-existing lipid peroxides and the lipid peroxyl radical. Biochem J 352:27-36
13. Mozuraityte R, Rustad T, Storrø I (2006) Pro-oxidant activity of Fe^{2+} in oxidation of cod phospholipids in liposomes. Eur J Lipid Sci Technol 108:218-226
14. Mozuraityte R, Rustad T, Storrø I (2006) Oxidation of cod phospholipids in liposomes: Effects of salts, pH and zeta potential. Eur J Lipid Sci Technol 108:944-950
15. Kawakatsu M, Terao J, Matsushita S (1984) Phospholipid oxidation catalyzed by ferrous ion and ascorbic acid. Agric Biol Chem 48:1275-1279
16. Genot C, Kansci G, Laroche M (1994) Measurement of phospholipid oxidation in model membranes by determination of oxygen consumption with a semi-automatic polarographic method. Sciences des aliments 14:673-682
17. Chaiyasit W, Silvestre PC, McClements DJ, Decker EA (2000) Ability of surfactant hydrophobic tail group size to alter lipid oxidation in oil-in water emulsions. J Agric Food Chem 48:3077-3080

18. Bligh EG, Dyer WJ (1959): A rapid method of total lipid extraction and purification. Canadian J. Biochem 37:911-917
19. Kates M (1991) Techniques of lipidology isolation, analysis and identification of lipids. 2nd revised edition, Elsevier Science Publishers, The Netherlands.
20. International Dairy Federation (1991) International IDF Standard 74A. Belgium.
21. Ueda S, Hayashi T, Namiki M (1986) Effect of ascorbic acid on lipid autoxidation in a model food system. Agric Biol Chem 50:1-7.
22. Undeland I, Stading M, Lingnert H (1998) Influence of skinning on lipid oxidation in different horizontal layers of herring (*Clupea harengus)* during frozen storage. J Sci Food Agric 78:441-450.
23. McDonald R, Hultin HO (1987) Some characteristics of the enzymic lipid peroxidation system in the microsomal fraction of flounder skeletal muscle. J Food Sci 52:15-21
24. Fukuzawa K, Fujii T (1992) Peroxide dependent and independent lipid peroxidation: site-specific mechanism of initiation by chelated iron and inhibition by α-tocopherol. Lipids 27:227-233.
25. Ohyashiki T, Kadoya A, Kushida K (2002) The role of Fe3+ on Fe2+ - dependent lipid peroxidation in phospholipid liposome. Chem Pharm Bull 50:203-207.
26. Tadolini B, Gabrini L, Menna C, Pinna GG, HakimG (1997) Iron (III) stimulation of lipid hydroperoxide dependent lipid peroxidation. Free Rad Res 27:563-576.
27. Levenspiel O (1972) Chemical Reaction Engineering, 2nd ed, Wiley, New York.
28. Noguchi N, Yamashita H, Gotoh N, Yamamoto Y, Numano R, Niki E (1998).: 2,2'-azobis (4- methoxy -2, 4 –dimethylvaleronitrile), a new lipid-soluble azo initiator:application to oxidations of lipids and low-density lipoprotein in solution and in aqueous dispersions. Free Rad Biol Med 1998, 24:259-268.
29. Yoshida Y, Niki E (1992) Oxidation of methyl linoleate in aqueous dispersions induced by copper and iron. Arch Biochem Biophys 295:107-114.
30. Lide DR (2003) CRC Handbook of chemistry and physics. CRC Press, Boca Raton
31. Kawakatsu M, Terao J, Matsushita S (1984) Phospholipid oxidation catalyzed be ferrous ion and ascorbic acid. Agric Biol Chem 48(5):1275-1279.

Legends:

Fig. 1. Concentration of formed lipid peroxide (PV) and thiobarbituric reactive substances (TBARS) as a function of consumed oxygen during the oxidation of unsaturated fatty acids. The concentrations of Fe^{2+} and Fe^{3+} used to initiate oxidation were 7.5 μM or 15 μM. Phospholipid concentration in liposomes was 9 mg/mL. Each point represents an average of two determinations and standard deviation is given as error bars.

Fig. 2. Concentration of dissolved oxygen in liposome solution after addition of Fe^{2+} or Fe^{3+}. Each addition increased iron concentration by 7.5 μM. The phospholipid concentration in the liposome solution was 6 mg/mL.

Fig. 3. Decrease in concentration of dissolved oxygen $\Delta[O_2]$ observed immediately after addition of Fe^{2+} to a liposome solution (6 mg/mL) for different amounts of added Fe^{2+}.

Fig. 4. Changes in concentration of dissolved oxygen as function of time with low concentrations of phospholipids (a) and the change in the concentration of Fe^{2+} in the same experiments (b). Concentration of added iron was 15 μM Fe^{2+}.

Fig. 5. Changes in concentration of dissolve oxygen in liposome solution (6mg/ml) with different concentrations (0-400 μM or 0-66μmoles/g PL) of TPP. To catalyse the reaction 15 μM of Fe^{2+} was used.

Fig. 6. Changes in Fe^{2+} concentration in liposomes (6mg/mL) after addition of 2,2–dipyridyl a) 2,2-dipyridyl was added to liposome solution before addition of Fe^{2+} b) 2,2-dipyridyl was added to liposome solution after different oxidation periods (0, 10s and 5 min). The oxidation was induced by 15μM of Fe^{2+}. The increase in Fe^{2+} concentration during the first 20 min after addition of 2,2-dipyridyl was used for calculation of the rate of Fe^{3+} reduction.

Fig. 7. Proposed mechanism for oxidation of unsaturated fatty acids.

Fig. 8. Changes in concentration of dissolved oxygen in liposome solutions (6mg/mL) with different concentrations (0-250 μM, 0-42μmoles/g PL) of DPPD. To catalyse the oxidation was used Fe^{2+} or Fe^{3+} at 15 μM.

Fig. 9. Changes in dissolved oxygen concentration $[O_2]$, peroxide [PV] and [TBARS] as a function of time. Phospholipid concentration in liposomes was 9 mg/ml, and 7.5 μM of Fe^{2+} was used to catalyse oxidation.

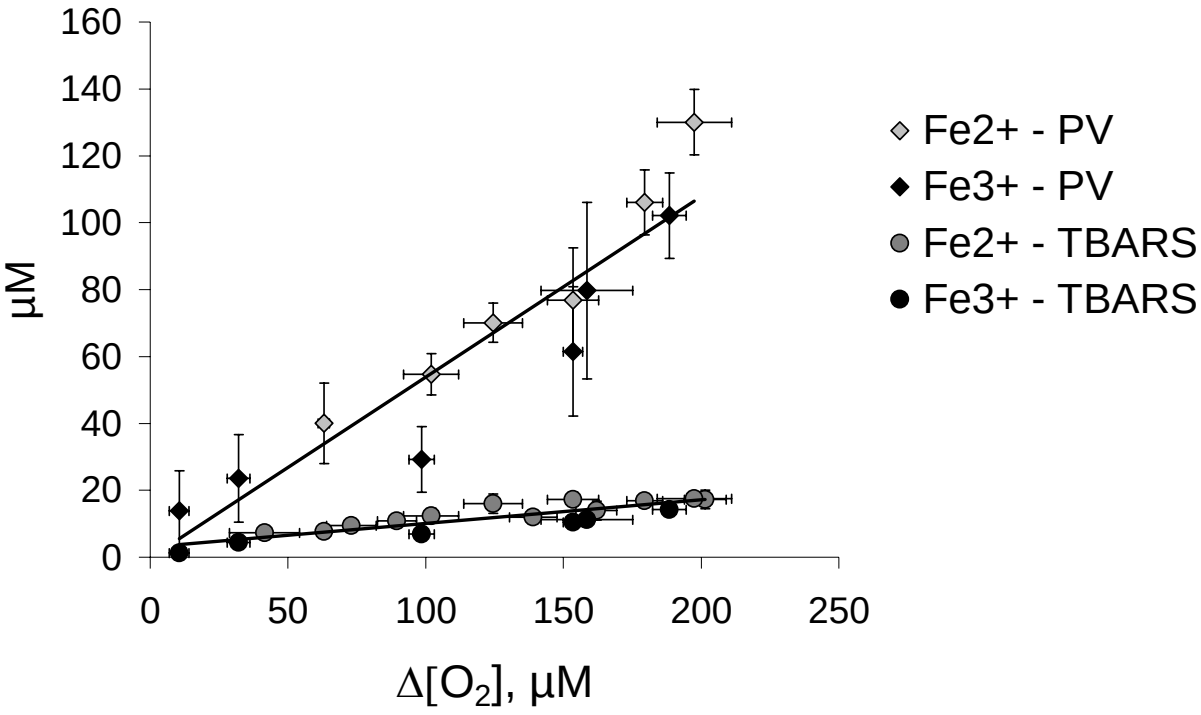


Fig. 1. Mozuraityte et al.

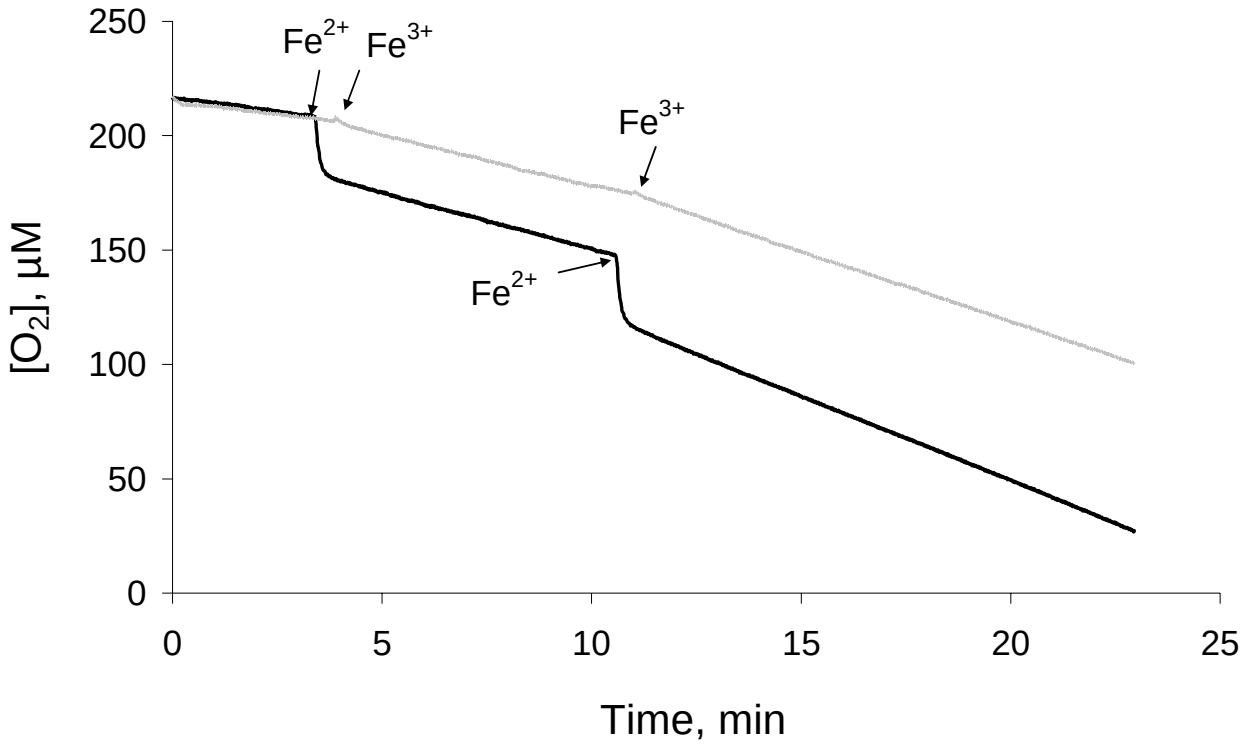


Fig. 2. Mozuraityte et al.

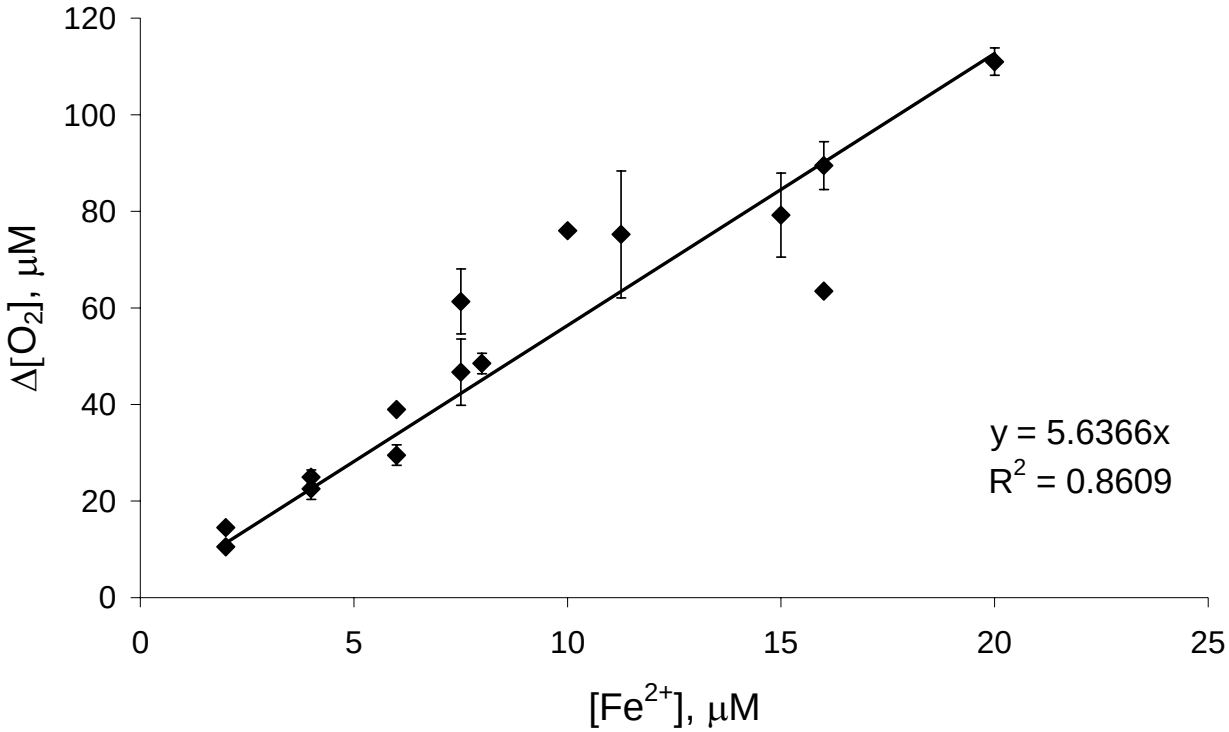


Fig. 3. Mozuraityte et al.

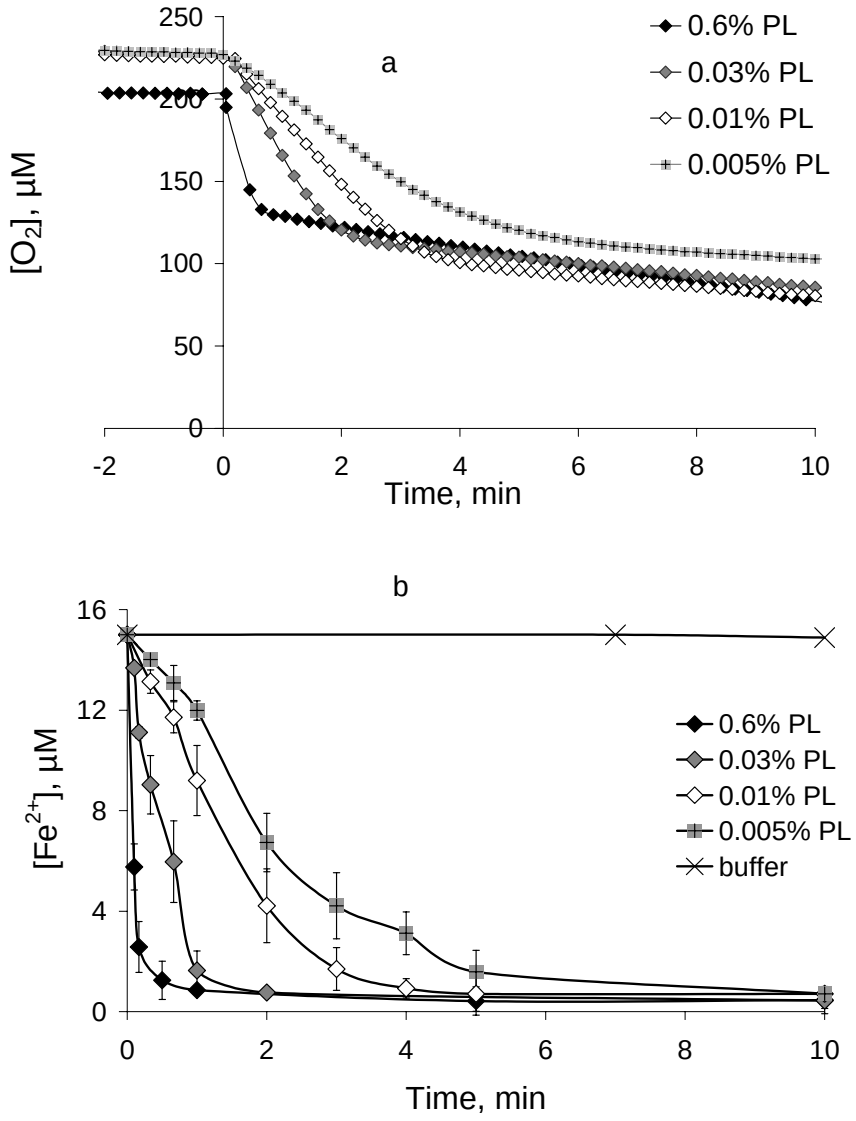


Fig. 4 Mozuraityte et al.

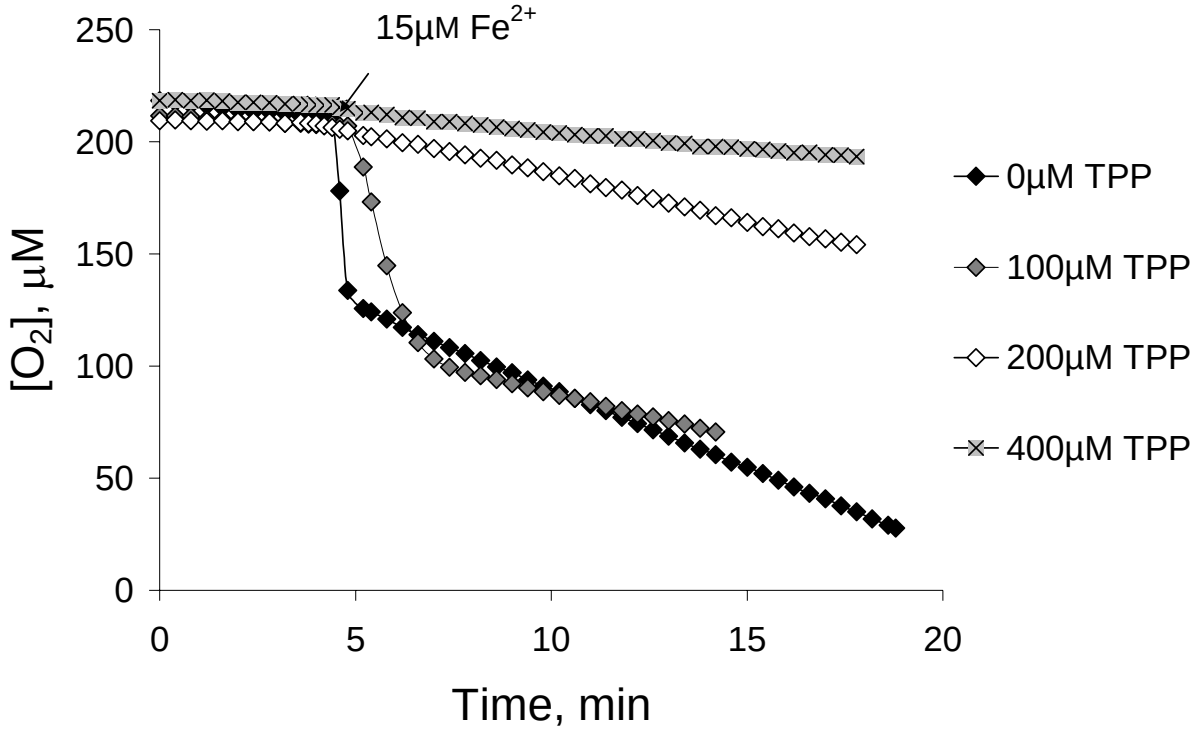


Fig. 5.Mozuraityte et al.

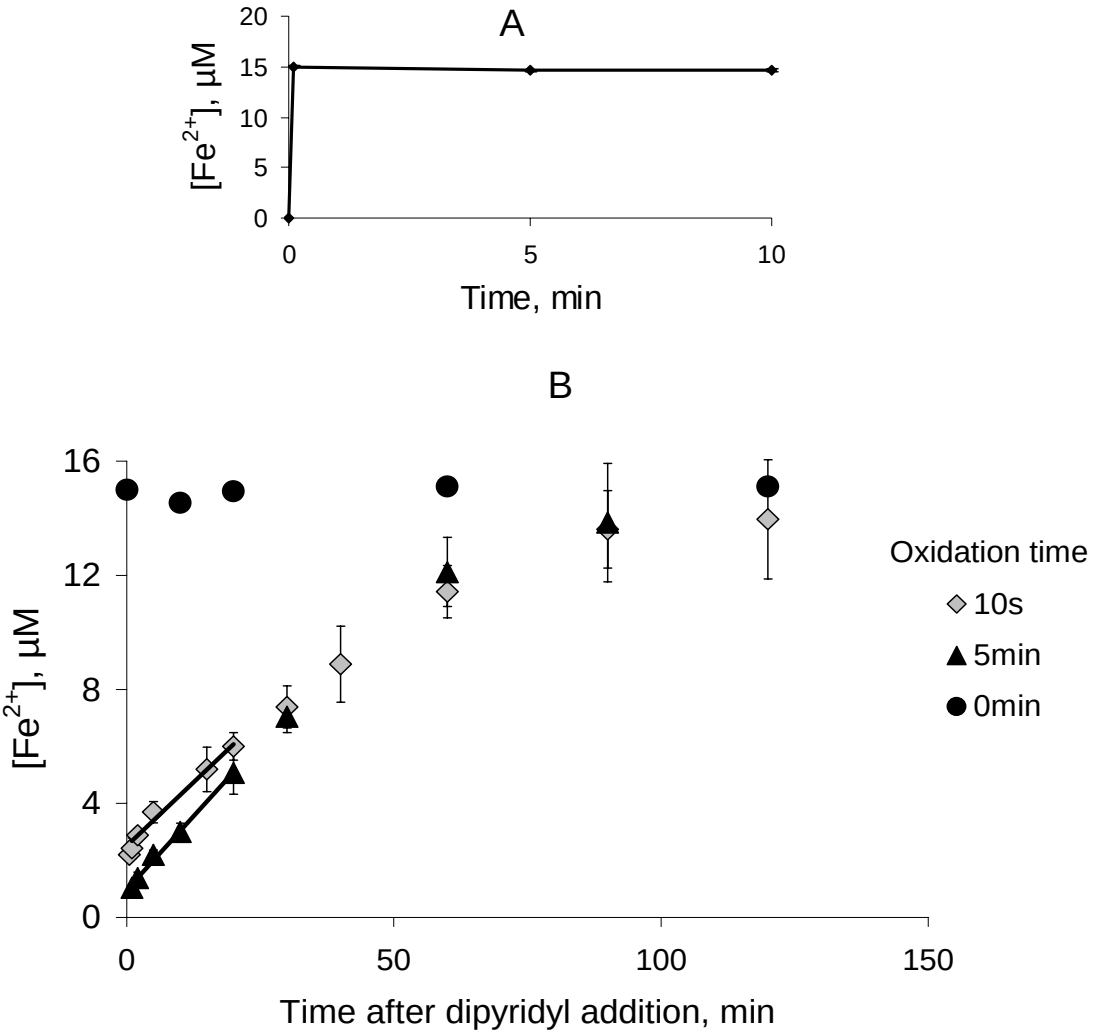


Fig. 6. Mozuraityte et al.

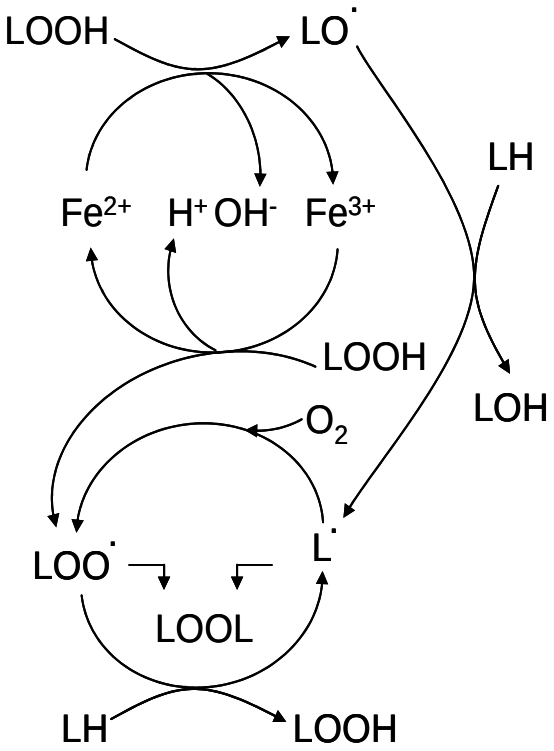


Fig. 7. Mozuraityte et al.

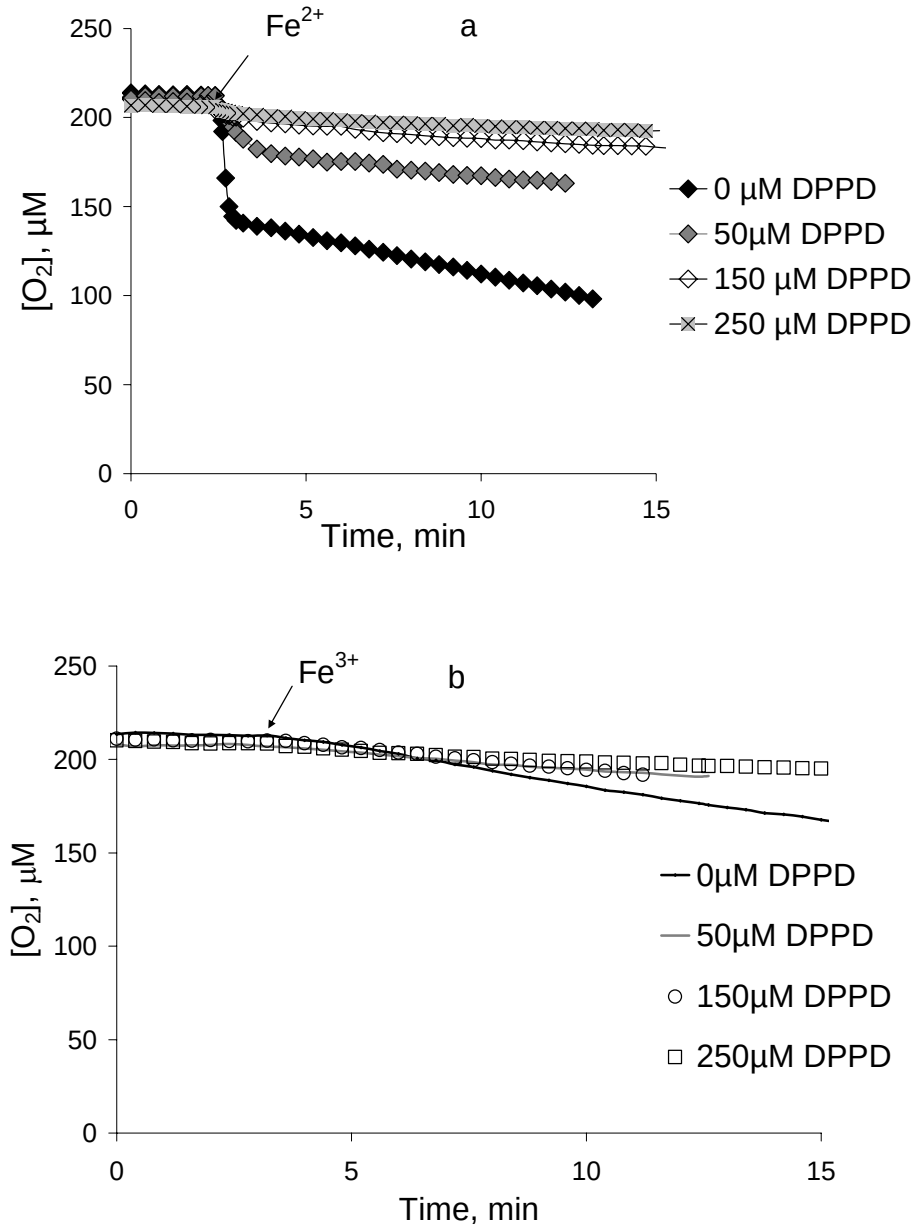


Fig. 8.Mozuraityte et al.

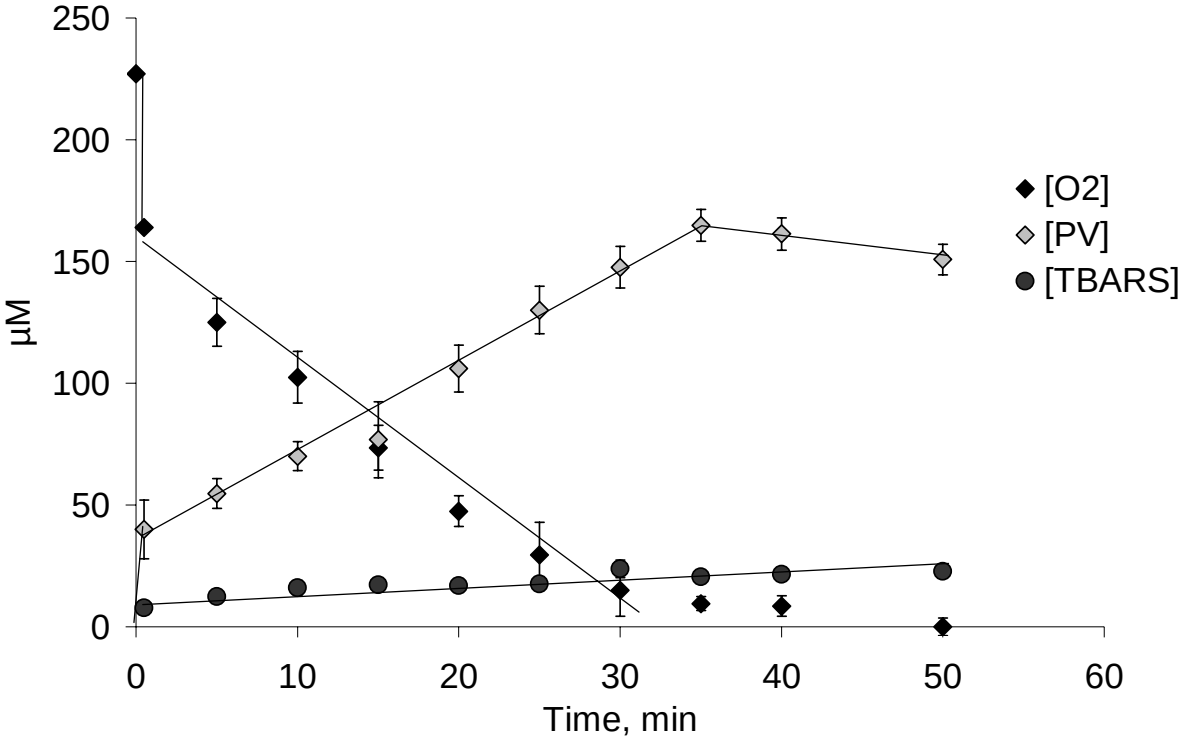


Fig. 9. Mozuraityte et al.

Table 1.

Concentration of lipid peroxides and the rate of oxygen uptake and Fe^{2+} oxidation during the initial drop in dissolved oxygen observed upon addition of Fe^{2+} in liposomes with different phospholipid (PL) concentrations. 15μM of Fe^{2+} was used to catalyse oxidation was used 15 μM of Fe^{2+}. Results are means ± SE. n=3

Concentrations		Rates	
[PL], %	[PV], μM	O_2 uptake, μM/min	Fe^{2+} oxidation, μM/min
0.600	36.2 ± 4.1	247.0 ± 21.2	75.1± 6.2
0.030	1.8	68.1 ± 0.6	12.8 ± 0.8
0.010	0.6	39.2 ± 2.8	5.4 ± 0.8
0.005	0.3	27.4 ± 0.9	4.2 ± 0.3
0	0	0	0

Table 2. The concentration of Fe^{2+} and the rate (r2) of production of Fe^{2+} from the reduction of Fe^{3+} at 20°C and 30°C measured as function of different times of oxidation of fatty acids in liposomes. Fe^{2+} was measured as 2,2–dipyridyl-Fe^{2+} complex. The rate constant was determined from the following equation: k2=r2/[Fe^{3+}]. The concentration of Fe^{3+} is given by total added Fe minus Fe^{2+}. Lipid concentration in the liposomes was 6mg/ml.

Time (t) s	T=20°C			T=30°C		
	$[Fe^{2+}]_t$, µM	r2 µM/min	k2 min^{-1}	$[Fe^{2+}]_t$ µM	r2 µM/min	k2 min^{-1}
10	2.504	0.178	0.014	1.932	0.254	0.019
30	1.555	0.198	0.015	1.805	0.268	0.020
60	1.053	0.227	0.016	1.634	0.256	0.019
300	0.968	0.207	0.015	1.368	0.295	0.022

Table 3. The rate of linear oxygen uptake (OUR) after two repetitive additions of iron. (7.5μM). Oxygen uptake rate per mole of iron ($\frac{OUR}{[Fe]}$) are means ± SE. n=4

	Fe^{2+}			Fe^{3+}		
[Fe], μM	7.5 μM[1]	15 μM[1]	15 μM[2]	7.5 μM[1]	15 μM[1]	15 μM[2]
OUR, μM/min	2.5±0.3	5.2±0.4	2.8±0.2	2.2±0.2	3.9±0.2	0.4±0.1
$\frac{OUR}{[Fe]}$	0.33±0.05	0.35±0.03	0.19±0.01	0.30±0.04	0.26±0.01	0.03±0.01

[1]- iron working solution at pH≈2
[2]- iron working solution at pH≈3.5

Paper III

Paper III is not included due to copyright.

Paper IV

The use of experimental design methodology for investigating a lipid oxidation rate assay

Martin Høy[a], Revilija Mozuraityte[b,d], Vegard Segtnan[a], Ivar Storrø[b], Bjørn Helge Mevik[c], Turid Rustad[d] and Tormod Næs[a,e].

[a] Matforsk, Norwegian Food Research Institute, Ås, Norway
[b] SINTEF Fisheries and Aquaculture, Trondheim, Norway
[c] Norwegian University of Life Sciences, Ås, Norway
[d] Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway
[e] Dept. of mathematics, University of Oslo, Oslo, Norway

1. Introduction

The area of experimental design comprises a number of useful techniques to be used for enhancing information content and reducing cost in experimental situations in science and technology (Box et al. 1978). The methods are relatively easy both to understand and to use, and a large number of successful applications have been reported. Typical examples are industrial process optimisation and product development (Myers and Montgomery 1995), but important examples exist in many other areas such as for instance consumer science, sensory analysis and optimisation of experimental conditions and instrument settings (Wortel et al. 2001).

The use of experimental design methodology always starts with careful consideration of which factors are of interest for investigation and at what levels these factors should be investigated (Box et al.1978). This should ideally be done in close collaboration between the statistician responsible for the experimental design and the investigator responsible for the subject matter under study. The size of the design is determined by the resources available. This can in practice vary from a small number of experimental runs, for instance 4 if only 2 factors are of interest to investigate, to more than hundred if the number of factors is large and the resources allow. In many cases it is recommended to do series of experiments in sequence. Starting out with a screening of all the factors involved followed up with one or several rounds of experiments of the most important factors. This is best done in the area of main interest identified in earlier rounds of experimentation. As soon as the experiments are performed the data are analysed with some type of analysis of variance or regression technique. In cases where several responses are of interest it is usually necessary to use more advanced methods like for instance PLS (Martens and Næs 1978) and 50-50 MANOVA (Langsrud 2002). Such methods can be used for building models over the region of interest and to assess the relative importance of the different experimental factors.

The present paper is a case study of the use of experimental designs for the purpose of investigating the effect of various factors on lipid oxidation rate. The main goal of the study was to gain insight into how the various factors interact and influence the response, but some

focus is also given to the signal to noise values. The former is important for future predictions of lipid oxidation rate under various experimental conditions while the latter is important for the purpose of identifying the most suitable regions for further data collection with focus on improvement of the model parameters of the assay. Results show that these problems can be solved by sensible use of a central composite design with repeated measurements of cube and centre points using analysis of variance (ANOVA) and partial least squares (PLS) modelling of the results. In this case, conducting a rather large set of experiments was possible and therefore no screening step was found necessary. As can be noted this problem has some similarities with so-called Taguchi robust design methodology (Kackar 1985, Vining and Myers, 1990).

A second important aspect of the present paper is to compare an empirical polynomial approach to one based on more detailed chemical and physical models, which is the traditional approach taken in such situations. In this way, an assessment is made of the advantages and disadvantages of the experimental design approach as opposed to benchmark methods in the area of chemical reaction optimisation. An empirical approach is used for the predictions obtained from the theoretical model for the purpose of getting information on which factors that are responsible for possible discrepancies.

2. Materials and methods

2.1 Experimental designs

The experiments used in the present paper are conducted in three series. In the first series, the only focus is on the shape of the response surface, and a full central composite design (with some points replicated) was used. In the second series, focus is on variability. A fractional factorial design with centre point, based on the same setting of the cube points as in the first series, was used and repeated a number of times for the purpose of estimating the signal to noise ratio. The third series was conducted in order to supplement the data in series two and to validate the results.

Originally, the central composite design was to be used for both response surface modelling and estimates of variability. But due to constraints in the experimental set-up the repeated experiments were not performed in the required, randomised order. This made it impossible to establish a fair estimate of the measurement error. Because of this, a separate set of experiments (the factorial design) had to be performed in order to estimate the variability.

The central composite design was based on the five design variables of interest. For the present experiment, these were pH (P), ionic strength (I), Fe^{2+} concentration (F), temperature (T) and lipid concentration (L). The variable levels used in the design are shown in Table 1.

Central composite designs are composed of two separate parts, the so-called cube points and the so-called star points. The cube points in this case correspond to a factorial design with two levels for the five factors and the star points correspond to the centre point(s) of the cube and centre points for the different plane surfaces defined by the other variables. Usually these star points are extended so that the distance from each experimental run to the centre is the same (rotational design). This is illustrated in Figure 1 for the case of two experimental factors. In this experiment, however, the star points were projected onto the cube in order to reduce the

number of levels for each factor from 5 to 3. This is termed faced central composite design. The central composite design is suitable for providing an experimental basis for second order polynomial modelling of the response as a function of the design variables. This first design consisted of 84 experimental runs since some of the samples were replicated.

In the second series a fractional factorial design (2^{5-1}) was used. Each of the 16 cube points from series 1 was repeated three times, to facilitate variance calculations. The experiments were conducted over 4 days. At the beginning and end of each day, two centre point experiments were run. This gives rise to a total of 16 centre points, and adds the possibility to check for instrumental drift within one day and between the days. In total, the second series of experiments thus consisted of 64 runs. For this series of measurements, two identical instruments were used to obtain the measurements. The 64 experiments were divided between the instruments in a systematic way in order to check for different instrumental responses.

For practical reasons, the randomisation had to be restricted with regard to temperature. Due to long temperature equilibration time in the instrumental setup, experimental temperatures were changed three times during a run of 8 samples. Number of samples within each temperature varied from 1 to 4. The first and the last run of the day were performed at centerpoint temperature, and between these two runs the temperature was changed twice in a randomized fashion.

The two first series of experiments were based on two different isolations on phospholipids, from which liposomes were prepared daily. The two series were compared in order to check for generalisation properties of the results. After the analysis of all these results, some of the samples were repeated, as will be described below, in order to improve the estimates for variability and for validation of the results. For this third series of experiments, yet another isolation of phospholipids was used. The different isolations of phospholipids were characterized with regards to chemical composition. No major differences were found by proton-, carbon- -NMR or by fatty acid analysis. Oxygen uptake, measured at standard experimental conditions, was the same for the different isolations. The same predictive relations given in equation (2)-(6) were used for the three phospholipid isolations.

2.2 Preparation of samples

Phospholipids used in the experiments were isolated from cod (Gadus morhua) roe. The fresh roe was removed from the roe sacks, frozen and kept at –40°C. Before extraction the roe was thawed in a cold room (+4°C) over night. The extraction of total lipids was performed according to the method of Bligh and Dyer (1959). Phospholipids were isolated from the total lipids using the acetone precipitation method (Kates, 1986), with a few modifications as described in a previous paper (Mozuraityte et al. 2006). The final precipitates (phospholipids) were stored in chloroform under nitrogen at –20°C until needed.

Background oxygen uptake rate was observed for 4-6 minutes before injection of iron as prooxidant. After injection of Fe^{2+} into the system, a fast decrease in dissolved oxygen was observed. This was followed by a slower linear decrease in dissolved oxygen, with a slope giving the rate of total oxidation. The oxidation rate was found by subtracting the background oxygen uptake rate from the total oxidation rate.

2.3 Analysis of experimental data

The data in the first series of experiments were analysed using a second-degree polynomial, with two-factor interaction terms and square terms included. The variables were centred before computation of interactions and square terms. This gave a model with 5 linear terms, 10 interaction terms and 5 quadratic terms. Regular Least Squares (LS) regression was used as a fitting criterion. The significance of the various effects was evaluated by F-tests. The validity of the model was determined by F-tests, cross-validation and plots of measured vs. predicted values of the response. The LS prediction results (using RMSEP, see Martens and Næs 1989) were also compared with PLS fitting of the data, but since the design in this case was orthogonal, the two methods yielded very similar results. But the results from the PLS model gave additional information about the samples and were e.g. used to identify outliers.

Since the response values (v) were unevenly distributed with most of the samples having a lower response value, a separate analysis was run with the response values log-transformed. The results gave little new insight and the transformation complicates the interpretation since the scale is different. In addition, the models from this approach were not significantly better when transformed back to original units than when using the original scale. The log-transformation was therefore not used in the rest of the analysis.

The residual and leverage of each observation was analysed, and outlying observations were removed before further modelling. In the first series of data, two observations were removed due to high leverage. Significance tests were used for the purpose of comparing models and for removing non-significant effects from the model.

For the second series of experiments, the standard deviation and mean value of the response were calculated for each experimental setting. Dividing the mean value by the standard deviation gives the signal to noise ratio (SNR).

Prior to the analysis of the standard deviation, a check for differences between the days and the two instruments were run. The results from this test, using regression analysis, showed that there were no significant differences neither between the days nor between the instruments. Thus, there was no need to adjust the variance estimates.

2.4 Traditional approach to calculations of individual response values

According to normal practice in the area, the theoretical models are built using a sequential multiplicative strategy.

The oxidation rate is defined as:

$$v = f(L) \times f(T) \times f(F) \times f(I) \times f(P) \tag{1}$$

where f(L) is a function for the influence of lipid concentration (L). Three linear regions have been identified for the relationship between L and v, making f(L) a decision tree function:

$$f(L)=\begin{cases} L\times 49.17 & \text{if} \quad L\le 0.03487 \\ L\times 3.8475+1.5814 & \text{if} \quad 0.03487<L<1 \\ 5.5 & \text{if} \quad L\ge 1 \end{cases} \tag{2}$$

The influence of temperature (T) follows the Arrhenius kinetics, where the reaction rate is dependent on the temperature in the following way:

$$f(T)=e^{\frac{-86456}{8.314}\times\left(\frac{1}{273+T}-\frac{1}{273+30}\right)} \tag{3}$$

The influence of the Fe^{2+} concentration (F) on the oxidation rate is derived experimentally, and is expressed as:

$$f(F)=\frac{0.54\times F}{0.54\times 7.5} \tag{4}$$

The effect of salt concentration or ionic strength (I) is also derived experimentally. The exponential decay equation is expressed as:

$$f(I)=\frac{1.2+5.7\times e^{-4\times I}}{1.2+5.7\times e^{-4\times 0}} \tag{5}$$

The influence of pH (P) is derived as:

$$f(P)=\frac{0.2706\times P^3-5.026\times P^2+28.706\times P-45.876}{0.2706\times 4.4^3-5.026\times 4.4^2+28.706\times 4.4-45.876} \tag{6}$$

The parameters in equation (2)-(6) were determined on the basis of experiments published elsewhere (Mozuraityte et al. 2006). For determination of the parameters in each equation, the variable of interest was varied in small steps while all the other variables were kept constant. Each of these data series was then used to fit the different nonlinear equations. The temperature dependency (equation 3) is based on chemical kinetics theory, while the other equations are purely empirical based on the observed data series. The modelling approach in equation (1) will in this paper be called the traditional approach.

3. Results and discussion

3.1 Effects and response surface

The ANOVA results for the regression of the first data series are summarised in Table 2. The model was calculated after removal of two clear outliers where the measured response values were very different from the other samples. The table shows that the linear, interaction (after fitting of linear terms) and square terms (after fitting of linear terms) are all highly significant. The square effects after fitting of linear and interaction terms are, however, only slightly

significant (p=0.037). Looking at the results in more detail, only one of the square terms (ionic strength) is significant, while all the interaction terms are significant at a 5% level. When used for making predictions, only the significant terms of the model were included.

The main and interaction effects of the raw data are illustrated in Figures 2a) and 2b) respectively. Note especially that the samples with zero ionic strength (top of first row) have a higher response than the other series, especially for certain values of the other variables. The interactions between ionic strength and the other factors seem to be the strongest.

Based on only the linear terms of the response surface model, the maximum response level is found at the highest temperature (T), Fe^{2+} concentration (F) and lipoprotein concentration (L), and at the lowest ionic strength (I) and pH (P). This conclusion is not changed by adding the significant interaction and square terms. As the response surfaces show (Figure 3), all maxima are found in the corners of each plot.

In Figure 4, the predicted values from the response surface model are plotted against the measured values. Prediction values from the soft model are from leave-one-out cross-validation. The response surface model performs reasonably well for the lower values, but slightly underestimates for the higher values. For comparison, the predictions from the traditional model are also included in the same figure. The most important thing to notice here is that the traditional model overestimates a lot for the highest values. The average prediction error for the calibrated soft model in this case was 0.98, while it was 3.23 for the traditional model. A more thorough comparison of the two will be considered below (Section 3.3).

3.2 Standard deviation and signal to noise ratio

The results from the second experimental design had three replicates at each experimental setting, and the results from this design were used to evaluate the total measurement error in the experimental area of interest.

In Figure 5 the standard deviation of the measurements is plotted against the mean response. The first thing to notice is that the standard deviation is around 10% of the measurement value, which is quite normal in this type of studies. The next thing to notice is that there is a weak tendency for the variance to increase with the response. But the results for the points with high response values are rather inconclusive, only three of five points suggest that the variance increases with the response. Due to this inconsistency, these five experiments were repeated 8 more times in order to get a better estimate of the standard deviation.

The third set of experiments was divided in eight blocks and run over two days using two instruments, with two blocks per instrument and day. For the third set of experiments, a new isolation of phospholipids was used. The re-estimated values of the standard deviation are plotted in Figure 6. The picture is now much clearer, as the tendency from the first results is confirmed.

The signal to noise ratio (SNR) is given by the mean response divided by the standard deviation. In Figure 7 the SNR is plotted against the mean response, showing clearly that the SNR is the same in the entire measured region. Thus, based on the SNR, one cannot argue that measurements within the observed area are more precise with a particular set of experimental settings.

3.3 Comparison of the two modelling approaches

The soft model developed from the first series of experiments was also used to make predictions for the second experimental design. Prediction results for the traditional model and the soft model are shown together in Figure 8. The average prediction error (RMSEP) was 2.24 for the traditional model, and 1.68 for the soft model. For this data set, the traditional model performed better than for the first data set, where the results were highly influenced by the samples that were vastly overestimated. However, it should be noted that the experimental conditions with the highest response values are not represented in this second set. The soft model performed somewhat poorer for this dataset, which is to be expected since the parameters were estimated from the first dataset. The increase in prediction error for the soft model approach may also indicate that the two different isolations of phospholipids behave slightly differently.

The soft model performs best for the middle points, while it underestimates slightly for the high and low values. But the centre-points in the design (around 4 in response) are very close to the target line. The traditional model underestimates all the low values including the centre-points, and overestimates most of the middle values. But the highest response values are estimated quite well. Note that the highest values in this series of experiments are not as high as in the first series, and thus the performance of the traditional model is also better. One could also note that the traditional model predicts the same response value for samples that have quite different measured values. This can be observed as horizontal patterns in the plot.

In order to try to pinpoint the shortcomings of the traditional model, a plot was constructed that compares the observed data (for experiment 1) with the predicted values from the model. The plot is created in the same way as an ordinary main effects plot: For each level of each design variable, the mean value of all observations measured with the selected level of the selected variable is calculated. Each subplot then shows the observed effect of each variable. The values for the two outlying observations were replaced by representative values before the mean value was calculated. In order to analyse the traditional model, the same procedure was followed, but this time with the response values predicted from the traditional model. In Figure 9 the behaviour of the traditional model can be compared with the actual observations, using data from the first experimental design.

The first thing to notice is that the overall mean (horizontal lines) of the estimates from the traditional model seems reasonably good. But looking at each of the plots, we can see that for low and especially middle values of the response, the model underestimates, while the model overestimates for the high response values. We can also see that underestimation at low values and overestimation at high values is a problem for all variables except pH. For the variable pH, the average predictions for the lowest and highest values are good, but the observed effect is more linear than the one implemented in the model.

These results indicate regions where the traditional model may be imprecise and where more detailed considerations must be undertaken in order to improve its properties. They also indicate possible directions of future modifications.

4. Conclusions

The effect of five variables on the rate of lipid oxidation was investigated using experimental design and response surface modelling. All the design variables, all of the two-factor interactions and one quadratic effect were found to be significant by the response surface model.

The measurement error in the experimental area of interest was estimated by repeated measurements, and based on this the signal to noise ration was calculated. The SNR turned out to be the same in all of the measured levels, indicating that the measurement error of the system is proportional to the signal level.

In addition to the empirical response surface model, a traditional approach based on more detailed chemical knowledge was used for fitting and for making predictions. The traditional approach consisted of both theoretically and empirically determined parts. When comparing the predictions, the response surface model showed a lower average prediction error than the mechanistic model. In order to look into the performance of the mechanistic model, the predictions from this model was plotted together with the measured values in a main effects (data means) plot. This gives insight into what parts of the mechanistic model that needs to be improved.

A different isolation of phospholipids was used for each experimental design. The models (both empirical and traditional) that were fitted to data from one experimental design still gave fairly good predictions when applied to new data generated from a second isolation of phospholipids.

Acknowledgements

This work was performed within the Integrated Research Project SEAFOODplus, contract No FOOD-CT-2004-506359. The partial financing of the work by the European Union is gratefully acknowledged.

References

Box, G.E.P., Hunter, W.G. and Hunter, J.S. (1978). *Statistics for experimenters.* John Wiley and Sons, NY.

Kackar, R.N. (1985). Off-line quality control, parameter design and the Taguchi method. *J. Qual. Techn.* 26 (4), 248-260.

Bligh, E. G. and Dyer W. J. (1959) A rapid method of total lipid extraction and purification. *Canadian J. Biochem.* 37, 911-917.

Kates, M. *Techniques of lipidology isolation, analysis and identification of lipids.* 2nd revised edition, Elsevier Science Publishers (The Netherlands) 1991.

Langsrud, Ø. Multivariate analysis of variance for collinear responses, *J. Royal Stat. Soc.: Series D*, 51(3): 305-317, 2002.

Martens and Næs(1989). *Multivariate Calibration.* John Wiley and Sons, Chichester, UK.

Myers, R.H. and Montgomery, D.C. (1995). *Response surface methodology.* John Wiley and Sons, NY.

Mozuraityte R., Rustad T., Storrø I.: Pro-oxidant activity of Fe2+ in oxidation of cod phospholipids in liposomes. Eur. *J. Lipid Sci. Technol.* 108 (2006) 218-226

Vining, G.G. and Myers, R.H. (1990). Combining taguchi and response surface philosophies: a dual approach. *J. Qual. Tech.* 22 (1), 38-45.

Wortel, V.A.L., Hansen, W.G. and S.C.C. Wiedemann (2001). Optimising multivariate calibration by robustness criteria, *J. Near Infrared Sepectroscopy.* 9, 141-151.

Figure 1. Illustration of central composite design

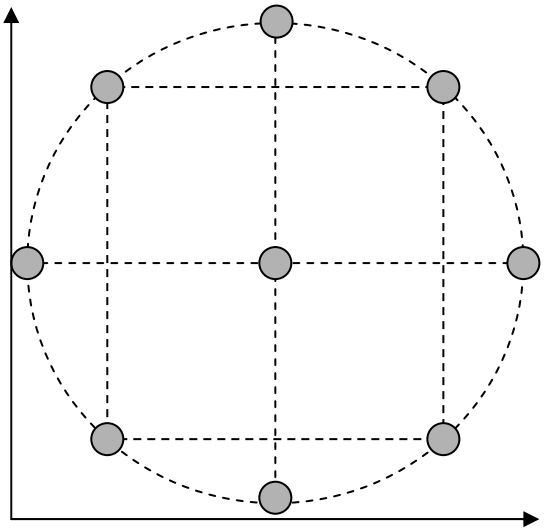


Figure 2a). Main effects plot (data means) for the response variable (v)

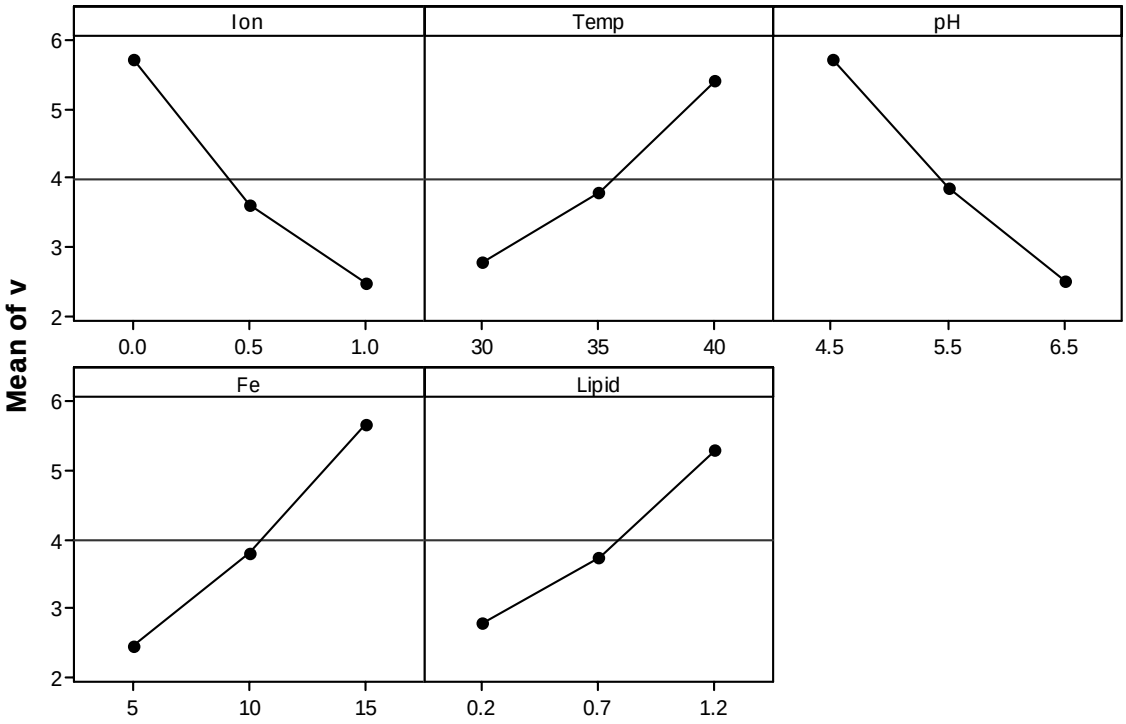


Figure 2b). Interactions effects plot (data means) for the response variable (v)

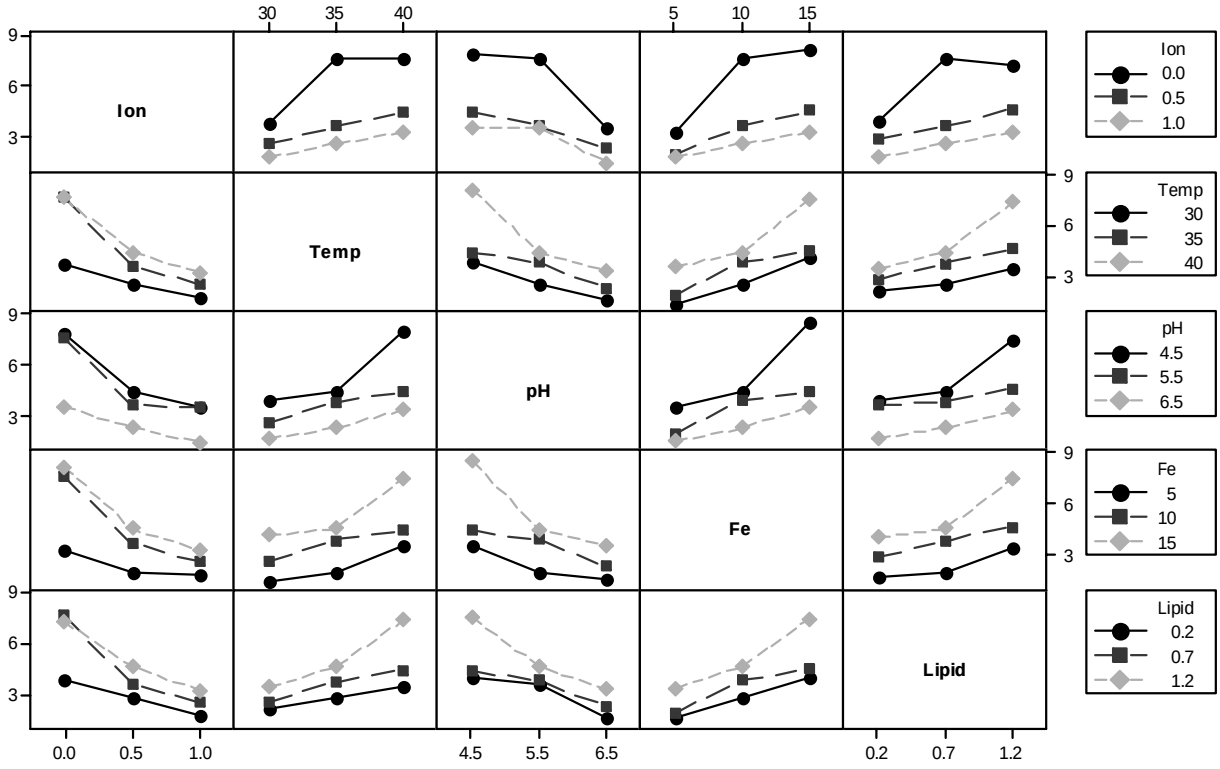


Figure 3. Contour plots for the response. Black dots indicate the design points for the middle settings.

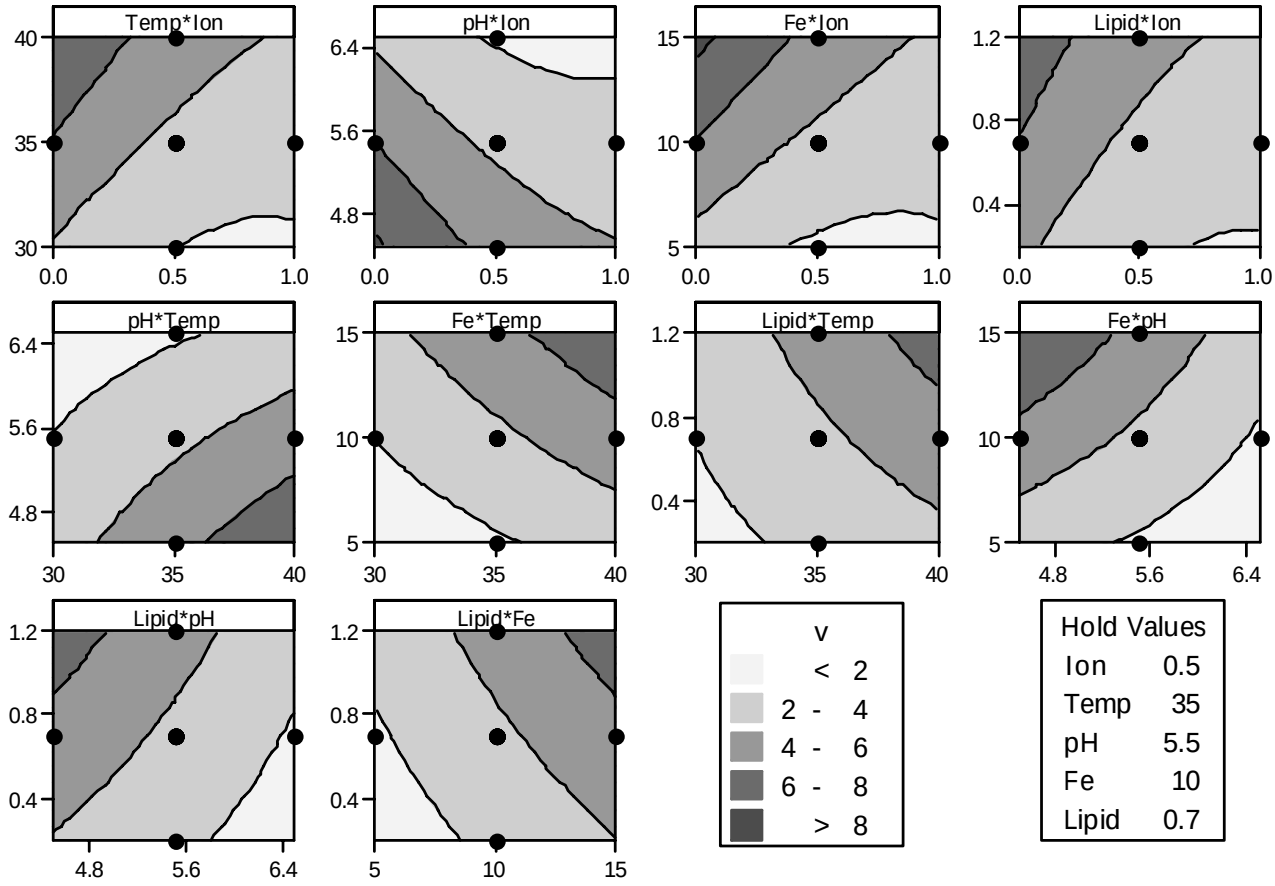


Figure 4. Predicted vs. measured values from the first series of experiments. Both the traditional and soft model predictions are shown. Predicted values from the soft model are from leave-one-out cross-validation.

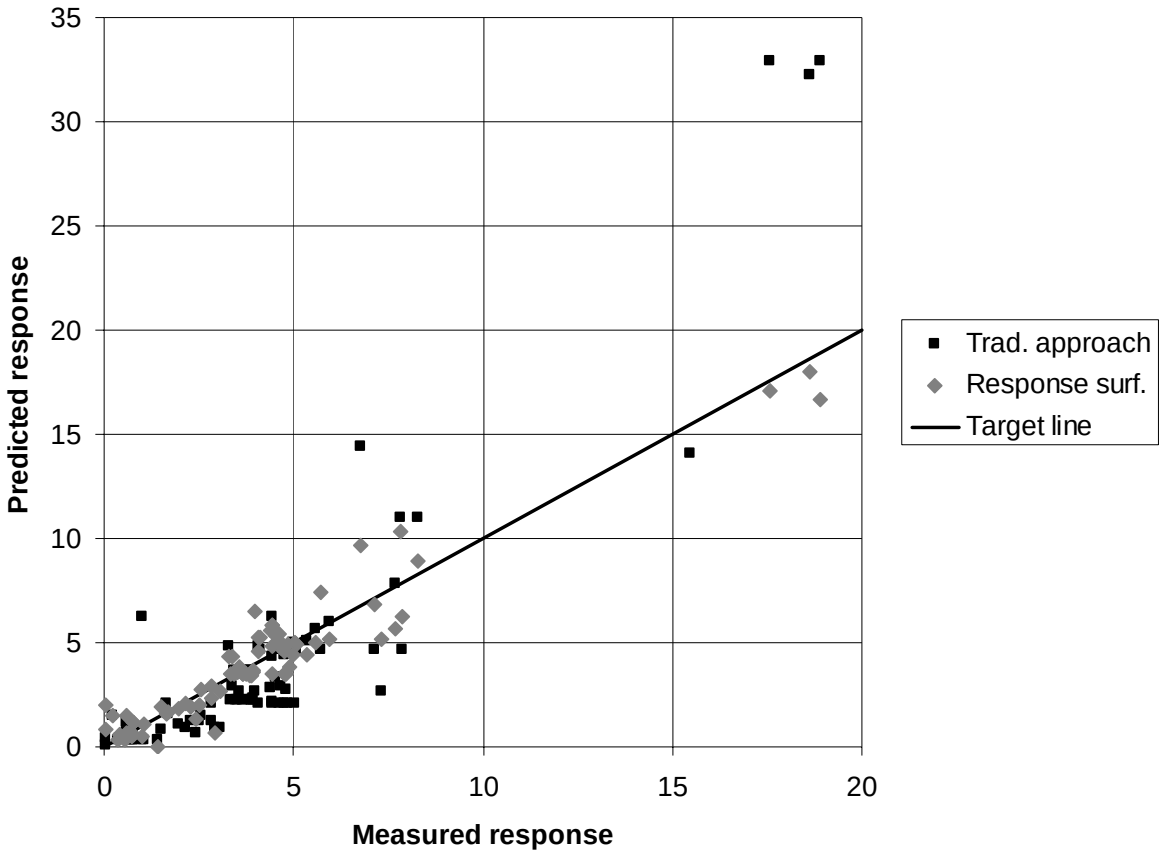


Figure 5. Standard deviation of measurements vs. mean response value.

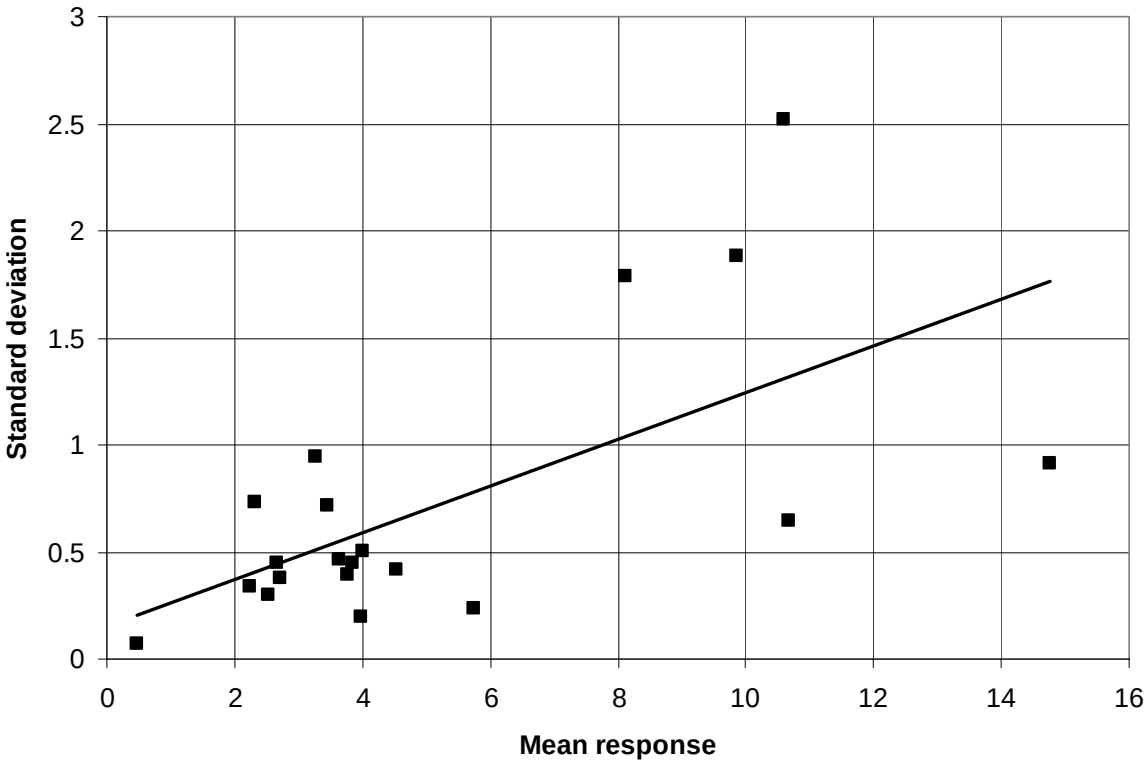


Figure 6. Standard deviation of measurements vs. mean response value, after extra replicates.

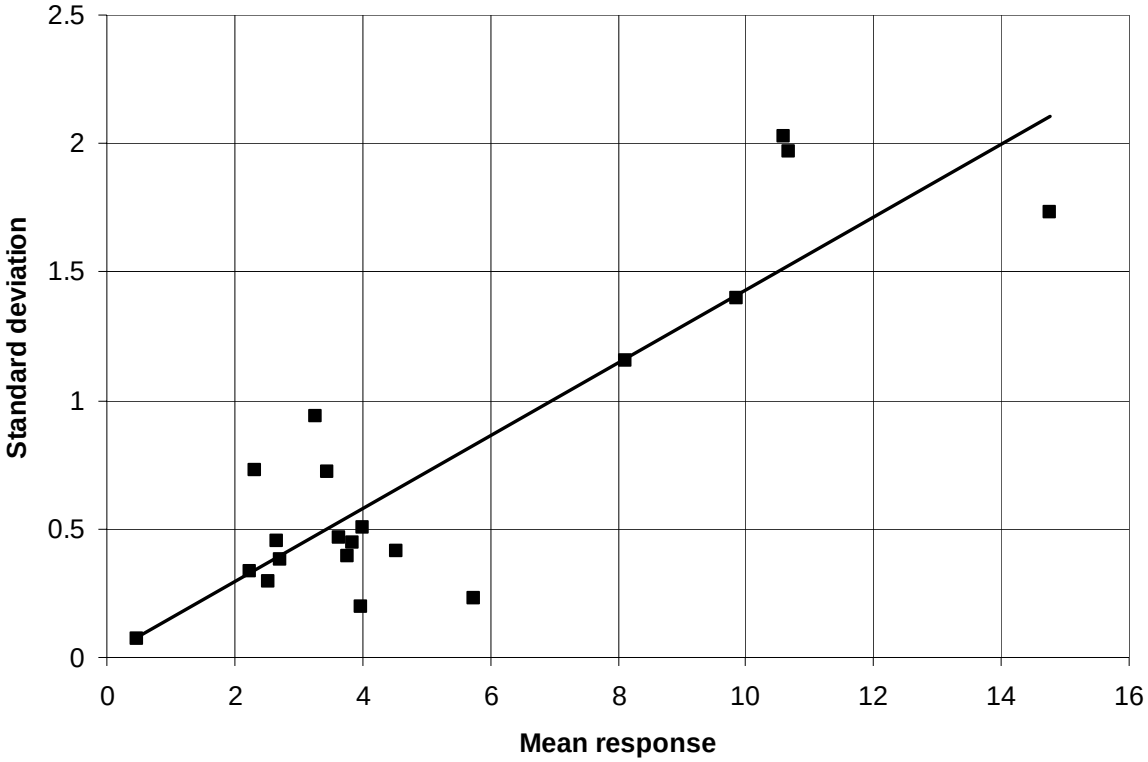


Figure 7. Signal to Noise Ratio (SNR) vs. mean response value, after extra replicates.

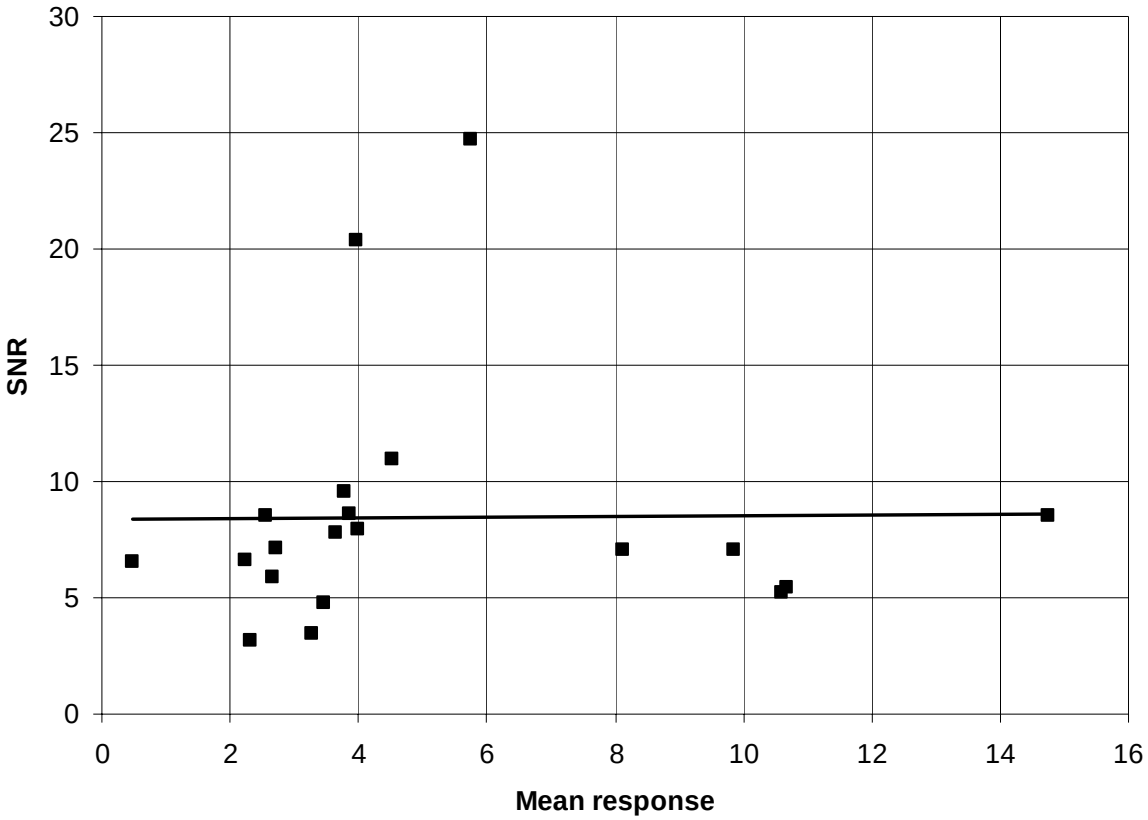


Figure 8. Prediction results for the traditional and soft model applied to the second series of data.

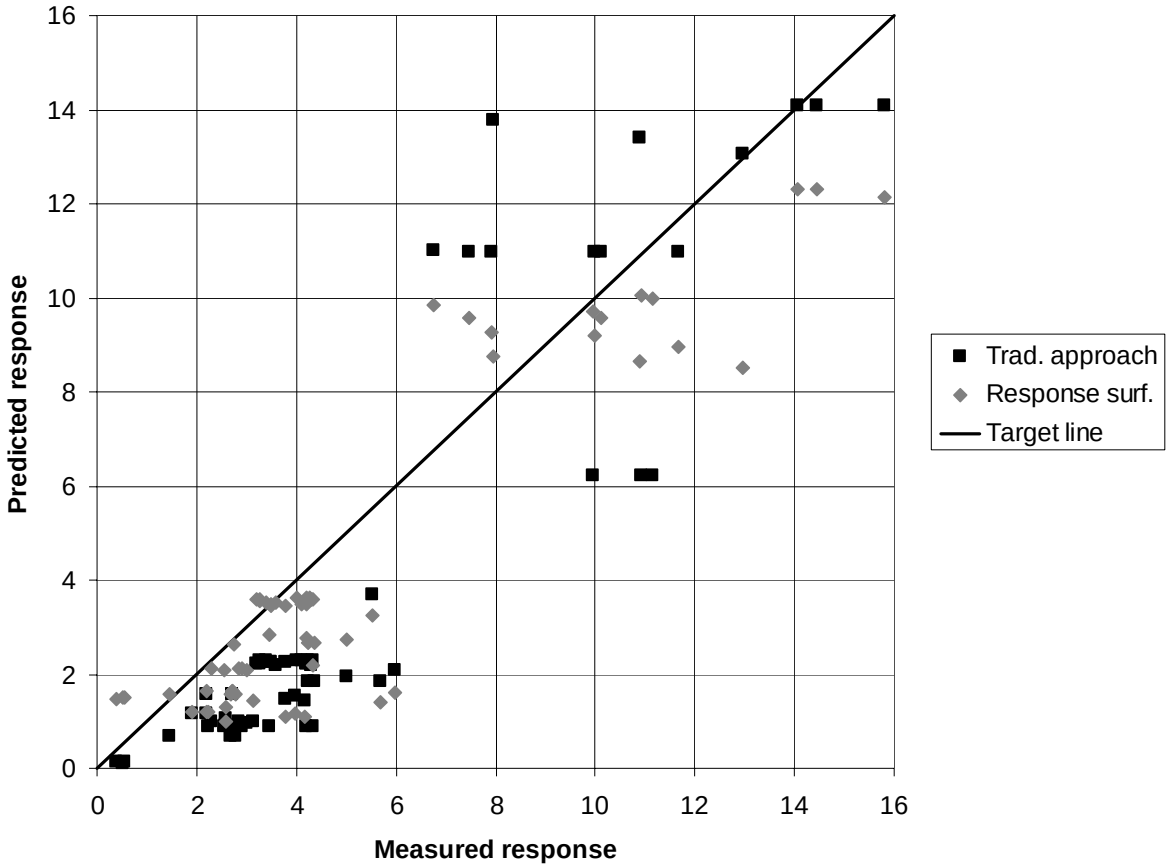


Figure 9. Main effects plot (data means) for the observed data and the predictions from the traditional model. The horizontal lines show the overall mean values of the observed and predicted values.

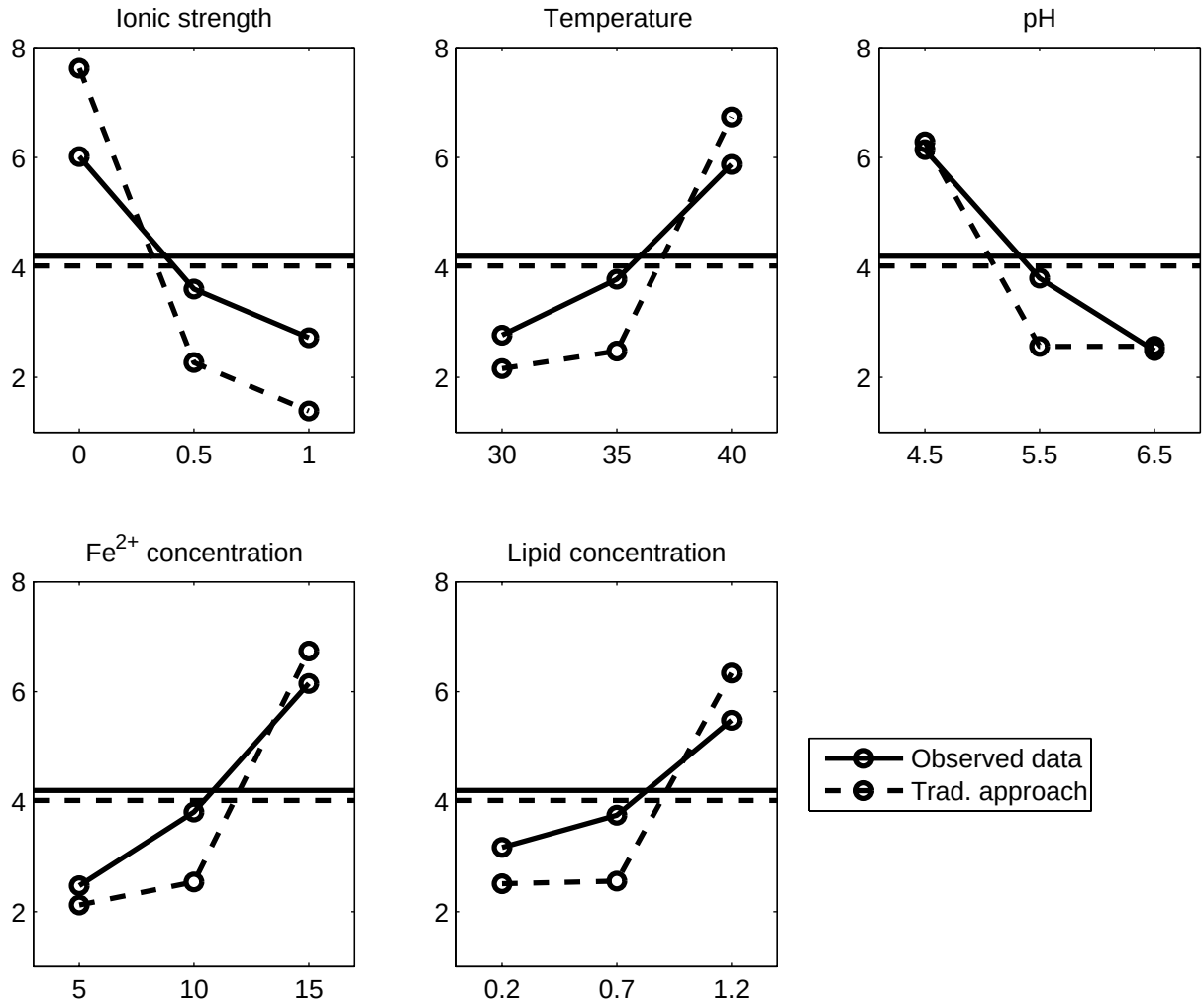


Table 1. Levels of the variables in the experimental designs.

pH	**Ionic strength (M)**	**Fe^{3+} (µM)**	**Temperature (°C)**	**Phospholipids (g/L)**
4.5	0.0	5	30	0.2
5.5	0.5	10	35	0.7
6.5	1.0	15	40	1.2

Table 2. ANOVA for the first series of experiments. The p-values for interaction and square correspond to p-values for the additional contribution after fitting of the linear terms. The Int+Square corresponds to the testing of the square terms after fitting of the interactions.

Source	**DF**	**p-value**
Regression	20	0.000
Linear	5	0.000
Interaction	10	0.000
Int + Square	5	0.037
Square	5	0.000
Residual error	61	
Total	81	